METABOLISM OF *S*-METHYLMETHIONINE AND ITS CONVERSION TO DIMETHYLSULFONIOPROPIONATE

By

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To my wife Connie,
and my sons
Rainer, Everit, and Ivan.

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LIST OF ABBREVIATIONS

AdoHcy	*S*-adenosylhomocysteine
AdoMet	*S*-adenosyl-L-Met
Ala	alanine
APS	adenosine 5'-phosphosulfate
Arg	arginine
Asn	asparagine
Asp	aspartate
ATP	adenosine 5'-triphosphate
BADH	betaine aldehyde dehydrogenase
Bis-Tris-HCl	bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane-HCl
Bis-Tris-propane-HCl	1,3-bis[tris(hydroxymethyl)-methylamino]-propane-HCl
CHES	2-(*N*-cyclohexylamino)ethanesulfonic acid
CoA	Coenzyme A
Cys	cysteine
DDH	DMSP-ald dehydrogenase
DMS	dimethylsulfide
DMSHB	4-dimethylsulfonio-2-hydroxybutyrate
DMSO	dimethylsulfoxide
DMSP	3-dimethylsulfoniopropionate
DMSP-ald	3-dimethylsulfoniopropionaldehyde
DMSP-amine	3-dimethylsulfoniopropylamine
DOX	DMSP-amine oxidase
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetate

FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GC	gas chromatography
Glc	glucose
Gln	glutamine
Glu	glutamate
Gly	glycine
GOX	glucose oxidase
HEPES-KOH	*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic) acid-KOH
HMT	homocysteine *S*-methyltransferase
HPLC	high-performance liquid chromatography
Ile	isoleucine
Leu	leucine
Lys	lysine
MALDI-MS	matrix-assisted laser-desorption ionization MS
MDH	malate dehydrogenase
MeOH	methanol
Met	methionine
methyl-THF	5-methyl-THF
MMT	Met *S*-methyltransferase
MOPS-KOH	3-(*N*-morpholino)propanesulfonic acid-KOH
MS	mass spectrometry
MTHB	4-methylthio-2-hydroxybutyrate
MTOB	4-methylthio-2-oxobutyrate
MTP-amine	3-methylthiopropylamine
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
Orn	ornithine
PAPS	3'-phosphoadenosine 5'-phosphosulfate

Phe	phenylalanine
PLP	pyridoxal 5'-phosphate
Pro	proline
SDC	SMM decarboxylase
Ser	serine
SMM	*S*-methyl-Met
TCA	trichloroacetic acid
THF	tetrahydrofolate
Thr	threonine
TLC	thin-layer chromatography
TLE	thin-layer electrophoresis
Val	valine

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METABOLISM OF *S*-METHYLMETHIONINE AND ITS CONVERSION TO DIMETHYLSULFONIOPROPIONATE

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Methionine (Met) *S*-methyltransferase (MMT) catalyses synthesis of *S*-methyl -Met (SMM) from Met and *S*-adenosyl-L-Met (AdoMet). The SMM can be reconverted to Met by transferring a methyl group from SMM to homocysteine, in a reaction catalyzed by homocysteine *S*-methyltransferase (HMT). The MMT and HMT activities together constitute the apparently futile "SMM cycle" and are found in all angiosperms. Thus SMM is a primary plant metabolite. In this study, insertional *mmt* mutants of Arabidopsis and maize were used to test the several hypotheses that have been advanced to account for SMM or its cycle. Both mutants lacked SMM and had no SMM cycle, but they grew and reproduced normally, ruling out a critical role for SMM as methyl donor or transport molecule. With Arabidopsis, the free Met pools were found to be unaltered in the mutant, but it had significantly higher AdoMet and lower *S*-adenosylhomocysteine levels than the wild type, and consequently a higher methylation ratio.

The secondary plant metabolite, 3-dimethylsulfoniopropionate (DMSP), is a compatible solute accumulated by certain angiosperms and many marine algae. The dicot *Wollastonia biflora* (Compositae) makes DMSP from SMM, whereas SMM is not involved in the algal DMSP pathway. Using radio- and stable-isotopic feeding techniques, it was shown here that the grass *Spartina alterniflora* also makes DMSP from SMM, but the pathway differs from that of the dicot. In fact, data suggested the existence of two novel enzymes in *S. alterniflora*, and that the DMSP pathway has therefore evolved at least three times. Data also suggested that one enzyme would decarboxylate SMM, and the other would convert 3-dimethylsulfoniopropylamine (DMSP-amine) to 3-dimethylsulfoniopropionaldehyde (DMSP-ald). Metabolic modeling indicated the second enzyme might have a much higher affinity for its substrate than the first. Specific radioassays were developed, and comparative biochemical examination showed the existence of an SMM decarboxylase (SDC) and DMSP-amine oxidase (DOX). The SDC is pyridoxal 5'-phosphate-dependent, strongly prefers the L-enantiomer of SMM, and has a high apparent K_m ($\approx$ 18 mM) for its substrate. The DOX requires O_2 for activity, shows an apparent K_m for DMSP-amine of 1.8 mM, and is not accompanied by DMSP-amine dehydrogenase or transaminase activity.

CHAPTER 1
INTRODUCTION

Where crops grow is strongly limited by their ability to deal with abiotic stresses (Boyer, 1982; Salisbury and Ross, 1985). Although many approaches have been devised for excellent pest management (Maloy, 1993; Strange, 1993; Ross and Lembi, 1999; Monaco *et al.*, 2002), currently there is no economical way to combat the non-biological stresses that limit crop growth. In this respect, the biosynthesis of 3-dimethylsulfonio-propionate (DMSP) is of interest because of the possibility that crop plants normally unable to make DMSP can be engineered to accumulate this sulfur-containing osmolyte and cryoprotectant (McNeil *et al.*, 1999). The biosynthesis of DMSP is also important because DMSP is the precursor of the gas dimethylsulfide (DMS), and the latter has major implications for the environment, including effects on weather patterns and climate (Charlson *et al.*, 1987; Malin, 1996). Although the DMSP and DMS produced by marine algae are more environmentally important on a global scale than DMSP and DMS from land plants, the saltmarsh grass *Spartina alterniflora* (smooth cordgrass) can be a locally important source (Steudler and Peterson, 1984; Dacey *et al.*, 1987; Aneja and Cooper, 1989).

Both DMSP and its breakdown product, DMS, can be thought of as resulting from secondary pathways that branch off from plant primary sulfur metabolism (Cooper and Hanson, 1998; Kocsis *et al.*, 1998; McNeil *et al.*, 1999). In all cases, the synthesis of

DMSP starts with the sulfur-containing amino-acid precursor, methionine (Met). Though plant sulfur metabolism is well understood up to and including the synthesis of Met and its activated form *S*-adenosyl-L-Met (AdoMet), the metabolism of Met itself, both primary and secondary, is poorly understood (Giovanelli *et al.*, 1980; Anderson, 1990; Hell, 1997; Azevedo *et al.*, 1997; Leustek and Saito, 1999; Amir *et al.*, 2002). This is especially true for the *S*-methyl derivative of Met, *S*-methyl-Met (SMM), a compound produced only by plants and present in all tissues of every angiosperm that has been examined (Mudd and Datko, 1986 & 1990; Pimenta *et al.*, 1998; Bourgis *et al.*, 1999; Ranocha *et al.*, 2001). Thus, SMM is a primary plant metabolite but only recently shown to be so. It is now known that in angiosperms DMSP is made from Met via SMM (Hanson *et al.*, 1994a, Kocsis *et al.*, 1998)

The ubiquitous angiosperm metabolite, SMM, participates in an apparently futile cycle in plants (Mudd and Datko, 1990; Bourgis *et al.*, 1999), the so called "SMM cycle" (Figure 1). In this cycle, Met is converted to SMM by an AdoMet-dependent methylation mediated by Met *S*-methyltransferase (MMT). The SMM is then reconverted to Met by donating one of its *S*-methyl groups to homocysteine in a reaction catalyzed by homocysteine *S*-methyltransferase (HMT) (Figure 1). Previous work has shown that MMT and HMT genes are expressed in all organs of Arabidopsis, and that the cycle is active throughout the plant (Ranocha *et al.*, 2001). Various hypotheses have been advanced to account for the presence of SMM or its cycle, but there is little experimental support for any of them (Giovanelli *et al.*, 1980; Mudd and Datko, 1990; Bourgis *et al.*, 1999, Ranocha *et al.*, 2001, Roje *et al.*, 2002). In the first phase of this work, *mmt* knockout

mutants were therefore sought and used to investigate the importance of SMM and its cycle (Kocsis *et al.*, 2003).

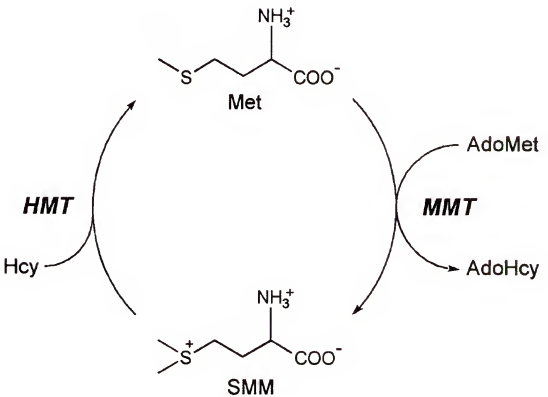

Figure 1. The SMM cycle of flowering plants shown in simple form. The reaction mediated by HMT generates two molecules of Met. Each revolution of the cycle uses one ATP. AdoHcy, *S*-adenosylhomocysteine; Hcy, homocysteine.

Although it is made from Met, SMM can also be considered the starting point for synthesis of the secondary metabolite DMSP in the higher plant *Wollastonia biflora* (dicot; Asteraceae/Compositae) (Hanson *et al.*, 1994a; Rhodes *et al.*, 1997). The DMSP is a potent osmoprotectant (compatible solute) that contributes significantly to osmotic stress tolerance of cells that accumulate it (Rhodes and Hanson, 1993; Hanson and Gage, 1996; Cosquer *et al.*, 1999). In using SMM as the starting point for DMSP synthesis, *W.*

biflora differs from marine algae, which use 4-methylthio-2-hydroxybutyrate (MTHB) (Gage *et al.*, 1997; Summers *et al.*, 1998). The second aim of this study was to check whether the *W. biflora* or algal-type pathway occurred in the coastal saltmarsh grass, *S. alterniflora* (Figure 2) . When it was found that the pathway was not the same as either the *W. biflora* or the algal one (Kocisis *et al.*, 1998), the next step was to identify the enzymes responsible for the novel pathway (Kocsis and Hanson, 2000).

Figure 2. Photograph of the saltmarsh grass *Spartina alterniflora*, also known as smooth cordgrass.
The C_4 grass, *S. alterniflora*, is one of a few salt-tolerant angiosperms which accumulate DMSP. This photograph was taken in the intercoastal waterway near Crescent Beach, Florida. Plant material was collected from the site, and used to initiate the greenhouse cultures employed in the experiments described in this study.

Comparative biochemistry combined with specific radioassays provided evidence for two novel enzymes specific to the DMSP pathway in *S. alterniflora*. The first novel enzyme is SMM decarboxylase (SDC). It is specific for the L- versus D-enantiomer of SMM, is pyridoxal 5'-phosphate (PLP)-dependent, and selective for SMM when compared to other amino acids which are structural analogs of SMM. The second novel enzyme is 3-dimethylsulfoniopropylamine (DMSP-amine) oxidase. It is a true oxidase, using molecular oxygen as a substrate. The enzyme is not a dehydrogenase; it does not use pyridine nucleotides as electron acceptors, nor is it dependent upon the addition of FAD or FMN. The chelated enzyme is somewhat stimulated by copper (Cu^{2+}) and, therefore, may be related to the ubiquitous copper-requiring topoquinone type enzymes (Janes *et al.*, 1992; Klinman, 1996).

Since *S. alterniflora* is important to local sulfur budget (Steudler and Peterson, 1984; Aneja and Cooper, 1989), understanding the biosynthesis of DMSP in *S. alterniflora* is important to understanding the biogeochemical sulfur cycle (Dacey *et al.*, 1987; Cooper and Hanson, 1998). Additionally, understanding DMSP biosynthesis in *S. alterniflora*, as well as the DMSP pathways in other plants, reveals enzymes whose encoding genes may be used in the metabolic engineering of crop plants resistant to abiotic (Cooper and Hanson, 1998; Nuccio *et al.*, 1999; Rontein *et al.*, 2002), and possibly biotic (Wolfe *et al.*, 1997), stresses. These abiotic stresses include drought, high-salt environments, and chilling as well as freezing (Low, 1985; Salisbury and Ross, 1985).

From an engineering standpoint DMSP is particularly interesting because, unlike quaternary ammonium compounds, tertiary sulfonium compounds do not impact nitrogen

budgets (Rhodes and Hanson, 1993; McNeil *et al.*, 1999). The application of fixed nitrogen derived by the Haber-Bosch process is one of the most important and expensive agricultural inputs. (In fact, at least as much nitrogen is fixed annually by this method than by all biological processes combined.) Additionally, from an engineering standpoint, DMSP pathways are attractive because they are unbranched and, in angiosperms, DMSP is accumulated but not further metabolized (Cooper and Hanson, 1998; Kocsis *et al.*, 1998; McNeil *et al.*, 1999).

CHAPTER 2
LITERATURE REVIEW

Synthesis of Sulfur Amino Acids in Plants

It is important to understand plant metabolism for two reasons. First, plants modify our environment (Taylor and Kiene, 1989; Turner *et al.*, 1989; Tolbert *et al.*, 1995). In addition to many other molecules, several volatile sulfur-containing compounds are emitted from plants that are thought to play a role in controlling weather, climate, and the pH of precipitation (Keller *et al.*, 1989; Rennenberg, 1991; Schröder, 1993; Hatton *et al.*, 1996; Malin, 1996). Second, we depend on plants nutritionally (Whitney *et al.*, 1990; Guthrie and Picciano, 1995). Plant metabolism is fundamentally different to that of animals, particularly mammals, in that plants are capable of the process of photosynthesis and, in general, they do not produce significant metabolic heat (Salisbury and Ross, 1985; Bowyer and Leegood, 1997). While using such inorganic compounds as carbon dioxide, water, nitrate, and sulfate, plants can harvest light energy from the sun and make all the organic molecules necessary for life. Because plants are immobile, relying on chemical defenses, and have been evolving since they first appeared at least 410 million years ago, they are diverse and both biochemically and genetically complex (Salisbury and Ross, 1985; Alberts *et al.*, 1989; Raven and Johnson, 1986).

Unlike animals (vertebrates), plants synthesize all of the amino acids they require. In fact, plants are the ultimate source of essential amino acids, of which methionine (Met)

is one of the most important. Animals (humans) must get Met in the diet, but since they make cysteine (Cys) from Met, when Met is missing from the diet there is no source of Cys either (Mathews and van Holde, 1990; Voet and Voet, 1995). In plants, Met is not only an important protein constituent, as in all organisms (Stryer, 1988; Hell, 1997), but is the precursor to compounds which are unique to plants (Gil and MacLeod, 1980; Giovanelli *et al.*, 1980; Yang and Hoffman, 1984; Anderson, 1990; Chapple *et al.*, 1990; Haughn *et al.*, 1991; Attieh *et al.*, 1995; Hanson *et al.*, 1997; Summers *et al.*, 1998; Graser *et al.*, 2000; Amir *et al.*, 2002). These include the important osmoprotectant and cryoprotectant, 3-dimethylsulfoniopropionate (DMSP), and the ubiquitous sulfur-containing phloem constituent, *S*-methyl-Met (SMM).

Of the 20 amino acids routinely incorporated into the proteins of all organisms, Met and Cys are the only two that contain sulfur. In addition to it being an important protein residue, biologically Met is very important as a precursor to the universal methyl donor and alkylating agent, *S*-adenosyl-L-Met (AdoMet). The AdoMet is, in turn, a precursor to the ubiquitous and volatile phytohormone, ethylene (Yang and Hoffman, 1984). In all organisms, Cys is used to make glutathione. Glutathione has been shown to be important in many biological functions, including control of redox potential, pathogen defense, sulfur transport, and others (Giovanelli *et al.*, 1980; Anderson, 1990; Bergmann and Rennenberg, 1993).

Unlike animals, which make Cys from Met, plants synthesize Met from the Cys that they make *de novo* (Giovanelli *et al.*, 1985; Anderson, 1990; Hell, 1997; Ravanel *et al.*, 1998). In general, sulfate is the primary and ultimate source of the sulfur atom in Met and Cys (Leustek and Saito, 1999). However, in a manner analogous to the uptake and

incorporation of nitrogen from nitrate, nitrite, and ammonium, it is possible sulfite and sulfide could also serve as an external source of sulfur to the plant cell (Giovanelli *et al.*, 1980; Anderson, 1990). The reduction of sulfate to sulfide requires eight electrons, twice as much energy as nitrate reduction to ammonium (Hell, 1997; Leustek and Saito, 1999). As with the other protein amino acids, the carbon skeletons of those that contain sulfur, and indeed all other organic molecules, are carbohydrate-derived (Azevedo, *et al.*, 1997).

Plant cells take up sulfate via proton-sulfate cotransporters (symport) embedded in the plasma membrane (Anderson, 1990; Hell, 1997; Leustek and Saito, 1999). Cells appear to have both high affinity and low affinity transporters (Hell, 1997; Leustek and Saito, 1999). High affinity transporters appear to be root-specific and may be important when the amount of sulfur in the environment is limiting to growth (Hell, 1997; Leustek and Saito, 1999). Once sulfate enters the plant cell it is incorporated into adenosine 5' -phosphosulfate (APS) via the activity of the enzyme ATP sulfurylase (also known as adenylylsulfate pyrophosphorylase). The reaction liberates pyrophosphate, and enzyme catalyzed hydrolysis of pyrophosphate "pulls" the reaction forward (Hell, 1997; Leustek and Saito, 1999).

Once sulfate is trapped in the cell in the form of APS, it needs to be reduced to sulfide in order for a sulfur atom to be incorporated into Cys (Giovanelli *et al.*, 1980; Anderson, 1990). In plants, the first step in this process is a two-electron reduction of APS catalyzed by APS reductase (Hell, 1997; Leustek and Saito, 1999). The reaction liberates free sulfite which, in turn, is reduced to sulfide. It is now known that the APS reductase reaction involves glutathione (Leustek and Saito, 1999). It appears that in some cases APS is further phosphorylated by ATP, via APS kinase, to yield a compound

found in *Escherichia coli* and other bacteria, 3'-phosphoadenosine 5'-phosphosulfate (PAPS). In this case it is PAPS that undergoes the two-electron, and apparently ferredoxin-dependent, reduction to yield sulfite. It is thought that the reduction of the sulfonyl moiety of APS in plants, and perhaps also that of PAPS in *E. coli*, to sulfite can occur by transfer of the sulfonyl group to a thiol acceptor. One possible acceptor is the vicinal dithiol protein, thioredoxin (Hell, 1997). This would yield $-S-SO_3$, and reaction with glutathione or a second thiol group on thioredoxin would yield sulfite and an oxidized acceptor.

The step for reducing sulfite to sulfide requires six electrons and, at least in plants, is ferredoxin-dependent (Leustek and Saito, 1999). The enzyme responsible, sulfite reductase, is a multisubunit complex composed of a protein containing both heme and iron-sulfur centers plus a flavoprotein (Mathews and van Holde, 1990; Hell, 1997). In *E. coli*, as in plants, the sulfite reductase catalyzed reduction of sulfite to sulfide occurs without the release of any free intermediates. In *E. coli*, PAPS can be converted back to APS through the activity of diphosphonucleoside phosphohydrolase. Bacterial APS is thought to be a storage form of sulfate. Recently it has been shown that plants have an additional pathway for converting sulfate to sulfide in the cell, and that it may resemble the pathway of bacteria (Hell, 1997).

The amino acid serine (Ser), which is derived from 3-phosphoglycerate, is a precursor to Cys (Giovanelli *et al.*, 1980). Through the activity of Ser acetyltransferase, acetyl-CoA (acetyl-Coenzyme A) is used to form the ester *O*-acetylserine, liberating CoA in the process (Anderson, 1990). The enzyme *O*-acetylserine (thiol) lyase (also called Cys synthase) catalyzes the reaction between sulfide and *O*-acetylserine that results in the

formation of Cys and acetate. The net effect of these two reactions is that the hydroxyl group of Ser has now been replaced with a sulfhydryl group, yielding Cys (Hell, 1997; Leustek and Saito, 1999). The reactions in most microorganisms are the same as in plants, although some bacteria appear to substitute succinyl-CoA for acetyl-CoA in the first reaction above, and some can condense Ser and sulfide directly to give Cys using a pyridoxal 5'-phosphate (PLP)-dependent enzyme. Vertebrates and other animals do not make Cys *de novo*. The Cys made by plants and microorganisms is incorporated into proteins, is used to make compounds such as glutathione, and most importantly is the precursor to the essential amino acid, Met (Giovanelli *et al.*, 1980; Ravanel *et al.*, 1998).

The sulfur amino acid, Met, is in the so-called "aspartate (Asp) family" of amino acids because Asp, which is itself an amino acid, is a precursor, as it is for the other amino acids in the family, threonine (Thr), isoleucine (Ile), and lysine (Lys). The Asp is derived from oxaloacetate from the tricarboxylic acid cycle. Thus, clearly the molecular skeleton of Met is carbohydrate derived. Via several well understood enzyme catalyzed steps, Asp is converted to *O*-phosphohomoserine (Giovanelli *et al.*, 1989; Brinch -Pedersen *et al.*, 1996; Azevedo *et al.*, 1997; Lugli *et al.*, 2000). The *O*-phosphohomo-serine is then condensed with Cys to yield cystathionine (Ravanel *et al.*, 1998). This reaction is catalyzed by cystathionine γ-synthase, a PLP-dependent enzyme (Ravanel, 1997). The reaction forms a covalent bond between the sulfur atom of Cys and the γ-carbon of *O*-phosphohomserine, freeing inorganic orthophosphate in the process. Cystathionine β-lyase breaks the covalent bond on the other side of the sulfur atom of cystathionine and results in the formation of homocysteine, pyruvate, and ammonium (Hell, 1997). Cystathionine β-lyase is also PLP-dependent (Ravanel, 1997; Ravanel *et*

al., 1998). Finally, Met is made from homocysteine by methylation of the sulfur atom of homocysteine through Met synthase (Anderson, 1990). In this case the methyl donor is methyl-THF (5-methyl-tetrahydrofolate). There is fairly strong evidence that, unlike the Met synthases found in animals and bacteria, the Met synthase activity of higher plants is cobalamin-independent (Giovaneli *et al.*, 1980; Ravanel *et al.*, 1998).

Clearly, in the *de novo* synthesis of Met by plants, it is not possible to use AdoMet as the methyl donor for the methylation of homocysteine, since AdoMet itself is derived from Met. As with all the carbon in organic molecules, the ultimate source of the methyl group put on to homocysteine, to make Met, is carbohydrate coming from the process of photosynthesis. (Even glycolate made during photorespiration, although not a carbohydrate, derives from the sugar ribulose bisphosphate whose synthesis is light driven.) To make Met, the Cys that is originally required to donate the sulfur atom comes from Ser (Giovaneli *et al.*, 1980). All but the methyl group portion of the Met carbon skeleton comes from oxaloacetate via Asp (Ravanel *et al.*, 1998). The methyl group on methyl-THF comes principally from from two amino acids, Ser and glycine (Gly).

In the first instance, when Ser is used as the source of the methyl group, the reaction is catalyzed by Ser hydroxymethyltransferase (Cossins, 1987; Appling, 1991). In the process Ser is converted to Gly and THF to $^{5,10}N$-methylene-THF (Hanson and Roje, 2001). Through the activity of $^{5,10}N$-methylene-THF reductase, and using NADH, $^{5,10}N$-methylene-THF is reduced to the ^{5}N-methyl-THF (i.e., methyl-THF) required for the methylation of homocysteine to make Met (Matthews *et al.*, 1998). Unlike the plant enzymes, which are NADH-dependent, the $^{5,10}N$-methylene-THF reductases of mammals and yeast are NADPH-dependent and feedback inhibited by AdoMet (Roje *et al.*, 2002).

In the second instance, the Gly resulting from the activity of Ser hydroxymethyltransferase or coming from other reactions serves as the substrate for the NAD^+-requiring enzyme, Gly decarboxylase (Cossins and Chen, 1997). Like Ser hydroxymethyltransferase, Gly decarboxylase also catalyzes the formation of $^{5,10}N$-methylene-THF, but liberates NADH, carbon dioxide, and ammonium in the process. Through the activity of $^{5,10}N$-methylene-THF reductase, the $^{5,10}N$-methylene-THF is reduced to methyl-THF which can be used for the methylation of homocysteine to make Met (Matthews *et al.*, 1998).

Plants, bacteria, and fungi can make homocysteine via transsulfuration reactions like those discussed above, and all organisms can make Met from homocysteine via Met synthase. Another biological transsulfuration process is conversion of homocysteine to Cys via the intermediate cystathionine (Mathews and van Holde, 1990). The enzymes involved are cystathionine β-synthase and cystathionine γ-lyase, and the process can be thought of as the reversal of the plant process involving the formation of cystathionine as an intermediate in Met formation (Stryer, 1988; Voet and Voet, 1995). In the formation of Cys from homocysteine, cystathionine β-synthase catalyzes a condensation reaction involving homocysteine and Ser to yield cystathionine. Cystathionine γ-lyase then catatalyzes the breakdown of cystathionine to release ammonium, α-ketobutyrate, and Cys. Although the cystathionine β-synthase and γ-lyase are found in animals and fungi, but not plants, it appears that plants may have the potential to incorporate sulfur derived from Met into Cys (Giovanelli *et al.*, 1980; Anderson, 1990).

AdoMet, SMM, and the SMM Cycle

As mentioned earlier, AdoMet is the universal biological methylating agent, and it is made from Met (Matthews *et al.*, 1998; Ravanel *et al.*, 1998). The high energy compound, AdoMet, is made by combining the adenosyl moiety of ATP with Met, via the activity of Met adenosyltransferase (also called AdoMet synthetase) (Lee *et al.*, 1997; Gómez-Gómez and Carrasco, 1998). In this reaction, the adenosyl group of ATP is transferred to the sulfur atom of Met, the covalent attachment being made at the adenosyl 5'-position previously occupied by the triphosphate group. This confers a permanent positive charge on the fully-substituted sulfur atom. This charge activates the ligands attached to the sulfur atom, making them good leaving groups, and accounts for the high transfer potential of AdoMet in relation to that of methyl-THF. Methyl-THF is thus used for relatively few methylation reactions (Giovanelli *et al.*, 1980). The Met adenosyltransferase activity results in the release of inorganic orthophosphate and pyrophosphate (Ravanel *et al.*, 1998). Thus, synthesis of AdoMet is somewhat special, since it requires the energy of two phosphoester linkages in the ATP molecule to make it.

Ubiquitous AdoMet is not only a source of methyl groups for many methyltransfer reactions, but it is also the precursor to other compounds which are partially derived from transfer of ligands, other than the methyl group, that are attached to the sulfur atom. For example, the ubiquitous polyamines, spermidine and spermine, result from transfer of the three-carbon moiety with its attached amino group from decarboxylated AdoMet to putrescine (Noh and Minocha, 1994). (Putrescine itself is made by decarboxylation of ornithine, an amino acid that is not one of the 20 typical protein residues). Polyamines are found in all living organisms, and appear to play important roles in many aspects of metabolism, growth, and development.

Furthermore, the important plant hormone, ethylene, is made from AdoMet via the unusual cyclic intermediate 1-aminocyclopropane-1-carboxylate (Yang and Hoffman, 1984; Wink, 1997). The enzymatic formation of this intermediate is PLP-dependent and thought to be the major control point in ethylene biosynthesis (Chae *et al.*, 2003). The 1-aminocyclopropane-1-carboxylate is made by intramolecular covalent attachment of the γ-carbon of the Met portion of AdoMet to the α-carbon, and then is enzymatically oxidized to yield cyanoformic acid (which breaks down spontaneously to HCN and CO_2) and, of course, ethylene. Therefore, ethylene represents or derives from carbons 3 (β) and 4 (γ) of Met.

The AdoMet that is found in all organisms is important for reasons mentioned above, and therefore it is expected that regulation of AdoMet level in the cell may be crucial. In plants, this is where the significance of SMM and the so called "SMM cycle" (Mudd and Datko, 1990) becomes manifest. Mudd and Datko (1986) were the first to observe in the small aquatic plant, *Lemna* (duckweed), that enzyme activities were present that allowed Met to be methylated by AdoMet to give SMM, and at the same time to use SMM to methylate homocysteine to give two molecules of Met. Recall that AdoMet is made from Met, and homocysteine itself is the result of the rapid enzyme -catalyzed breakdown of *S*-adenosylhomocysteine (AdoHcy), which is generated when AdoMet transfers its methyl group to any number of acceptors (Ravanel *et al.*, 1998). Therefore each turn of the SMM cycle consumes one ATP, and produces no new compound. This apparently futile cycle was suggested by Mudd and Datko (1990) to be a means of converting AdoMet back to Met so as to prevent an overshoot of AdoMet synthesis which, if unchecked, would bring protein synthesis to a standstill.

The SMM cycle has been found in all angiosperms investigated, and is unique to plants (Mudd and Datko, 1986 & 1990; Bourgis *et al.*, 1999; Ranocha *et al.*, 2001). It has a known role in sulfur transport (Bourgis *et al.*, 1999) and various other hypothetical roles, such as methyl donor, a way of stabilizing Met, and in AdoMet level control (Giovanelli *et al.*, 1980; Mudd and Datko, 1990). In Arabidopsis, there are three HMT genes but only one MMT gene (Figure 3). The three isoforms of the HMT protein differ in their K_m for SMM versus AdoMet, and in their sensitivity to Met (Ranocha *et al.*, 2000).

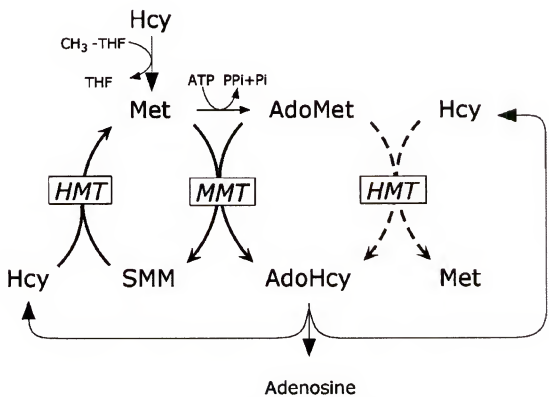

Figure 3. The SMM cycle and related reactions in flowering plants.
The two core reactions of the cycle are shown by unbroken arrows.

SMM as a Precursor of DMSP

There is evidence for an SMM → DMSP pathway in *Wollastonia biflora* (James *et al.*, 1995b; Trossat *et al.*, 1996), and evidence that marine algae have a completely different DMSP pathway, one that does not involve SMM (Gage *et al.*, 1997; Summers *et al.*, 1998). In angiosperms the first step in the conversion of Met to DMSP is the formation of SMM from Met (Kocsis *et al.*, 1998; Hanson *et al.*, 1994b). This is of interest since a clear role for SMM as anything other than a DMSP pathway intermediate has not been demonstrated, and yet it appears that all angiosperms, regardless of their DMSP biosynthetic ability, make SMM (Pokorny *et al.*, 1970; Mudd and Datko, 1990; Paquet *et al.*, 1995). The synthesis of SMM involves *S*-methylation of Met by Met *S*-methyltransferase (MMT), and uses AdoMet as the methyl donor (James *et al.*, 1995a; Hanson *et al.*, 1997). Additionally, angiosperms can convert SMM back to Met via methyl transfer from SMM to homocysteine, to yield two molecules of Met (Abrahamson and Shapiro, 1965; Dodd and Cossins, 1970; Mudd and Datko, 1990). This reaction is catalyzed by homocysteine *S*-methyltransferase (HMT), and its activity combined with that of MMT results in what Mudd and Datko (1990) have referred to as an apparently futile "SMM cycle" (Figures 1 and 3). These authors also suggested, however, that the SMM cycle may function under conditions of reduced methyl group requirement as a mechanism for the liberation of "trapped" methionine, needed for protein synthesis, from unnecessarily large AdoMet pools. At any rate, it is now apparent that SMM is ubiquitous in angiosperms.

Physiological Significance of DMSP

The accumulation of DMSP may be a good engineering target against abiotic stess because it is a tertiary sulfonium compound which is synthesized and accumulated by diverse photosynthetic organisms, including angiosperms (Paquet *et al.*, 1995; Hanson and Gage, 1996), marine algae (Keller *et al.*, 1989; Gage *et al.*, 1997), and, to some extent, phototrophic bacteria and cyanobacteria (Karsten *et al.*, 1996). DMSP is a sulfur -containing betaine analog that, like betaines, accumulates in cytoplasmic compartments of plant cells and functions as a compatible solute (Dickson *et al.*, 1980; Hanson and Gage, 1996; Trossat *et al.*, 1998). The role of DMSP as a potent osmoprotectant has been substantiated *in vivo* using bacterial bioassays (Paquet *et al.*, 1994; Summers *et al.*, 1998), and *in vitro* by demonstrating enzyme compatibility with high physiological concentrations of DMSP (Gröne and Kirst, 1991). Along with physiological evidence, enzyme protection assays indicate that DMSP also functions as a cryoprotectant (Nishiguchi and Somero, 1992; Karsten *et al.*, 1996).

DMSP differs from betaines and other quaternary ammonium osmoprotective compounds (Rhodes and Hanson, 1993) in that it does not contain nitrogen. In terms of agronomic importance, it is clear that an increase in stress resistance would be beneficial to most crops (Boyer, 1982). It is also significant, however, that DMSP accumulation does not negatively impact nitrogen availability for plant growth, since the amount of fixed nitrogen in soils is more often limiting to plant growth than any other element (Salisbury and Ross, 1985). In fact, DMSP appears to act as a replacement for betaines in environments where nitrogen levels are low relative to sulfur (Cooper and Hanson, 1998). The DMSP biosynthetic pathway is, therefore, a rational target for metabolic

engineering to increase abiotic stress resistance in crops, particularly in sulfur-rich, nitrogen-poor environments.

The three most important food crops worldwide are wheat, maize, and rice (Evans, 1993), none of which accumulate DMSP (Paquet *et al.*, 1994). Furthermore, when crops are ranked by weight of harvested commodity used for human or animal consumption the top five are gramineous species (Evans, 1993), but only one of these five, sugarcane, accumulates DMSP, and only in small amounts (Paquet *et al.*, 1994). Therefore, with respect to metabolically engineering DMSP accumulation into those crops from which it is likely the greatest benefits will be derived, it is necessary to understand DMSP biosynthesis and sulfur metabolism in grasses.

In the marine macroalga, *Enteromorpha intestinalis*, the pathway proceeds L-Met → 4-methylthio-2-oxobutyrate (MTOB) → 4-methylthio-2-hydroxybutyrate (MTHB) → 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB) → DMSP (Gage *et al.*, 1997; Summers *et al.*, 1998). The same pathway seems to operate in other DMSP-accumulating marine algae, including planktonic species representing major sources of DMSP and volatile dimethylsulfide (DMS) (Gage *et al.*, 1997). The compound DMSHB has been identified as a novel natural product and, so far, its synthesis appears to be unique to DMSP production in marine organisms. Assays have been developed for the enzymes which catalyze the pathway reactions, and the enzymes themselves, via activity measurements, have been characterized (Summers *et al.*, 1998). The *in vivo* activities associated with both the methyltransferase step and the step converting DMSHB to DMSP were shown to prefer the D-enantiomers of MTHB and DMSHB, respectively (Summers *et al.*, 1998). Additionally, DMSHB, which may be a precursor not only to DMSP but possibly to other

algal tertiary sulfonium compounds, such as gonyauline and 4-dimethylsulfonio-2 -methoxybutyrate, was shown itself to act as an osmoprotectant (Cooper and Hanson, 1998; Summers *et al.*, 1998). Algae can be a source of genes for the metabolic engineering of abiotic stress resistance in flowering plants.

In the dicotyledonous salt-tolerant plant, *W. biflora*, the DMSP biosynthetic pathway proceeds L-Met → SMM → 3-dimethylsulfoniopropionaldehyde (DMSP-ald) → DMSP (Hanson *et al.*, 1994b; James *et al.*, 1995b; Trossat *et al.*, 1996). The pathway has been well characterized and it was shown that DMSP accumulates in the chloroplasts of *W. biflora* in response to increased salinity (Trossat *et al.*, 1998). In *W. biflora* all the activities of the DMSP pathway after the formation of SMM occur in the chloroplasts (Trossat *et al.*, 1996).

In addition, the enzyme which catalyzes conversion of L-Met to SMM, i.e., MMT, has been purified and characterized (James *et al.*, 1995a), and the encoding gene has been cloned (Bourgis *et al.*, 1999). This will allow for the study of its role in the regulation of DMSP and the SMM cycle. Although the pathways in both *W. biflora* and algae start with the same primary amino acid precursor, L-Met, and end with the same product, DMSP, it is of major significance that these DMSP biosynthetic pathways have no intermediates in common (McNeil *et al.*, 1999). This suggests two independent evolutionary origins for the production of the same compound, from the same precursor, in response to similar types of osmotic stress associated with a high-salt environment.

Since most if not all angiosperms can make SMM (Giovanelli *et al.*, 1980; Bezzubov and Gessler, 1992), and many plants already contain at least one enzyme that can mediate the oxidation of DMSP-ald to DMSP (Trossat *et al.*, 1997; Vojtěchová *et al.*,

1997), it is therefore the conversion of SMM to DMSP-ald that is unique to DMSP synthesis. In *W. biflora*, it appears that the one-step mechanism by which SMM is converted directly to DMSP-ald involves either a transaminase-decarboxylase enzyme complex or an unusual enzyme that can carry out both the necessary steps (Rhodes *et al.*, 1997).

Immunological and kinetic evidence indicate that DMSP-ald dehydrogenase (DDH) and betaine aldehyde dehydrogenase (BADH), the enzyme that catalyzes the final step in glycinebetaine synthesis (Weretilnyk and Hanson, 1989 & 1990; McCue and Hanson, 1992), are one and the same (Trossat *et al.*, 1996 & 1997). Additionally, the DMSP pathway of higher plants seems to be, in every case for which there is information, superimposed on the glycinebetaine biosynthetic pathway, suggesting that BADH has been recruited into the DMSP pathways of angiosperms to catalyze the conversion of DMSP-ald to DMSP (Rhodes and Hanson, 1993; Paquet *et al.*, 1994; Colmer *et al.*, 1996). In this respect, it is significant that the step catalyzed by DDH has been shown in *W. biflora* to be located in the stromal compartment of the chloroplasts (Trossat *et al.*, 1996), in the same manner as is the major isozyme of spinach BADH (Weigel *et al.*, 1986; Rathinasabapathi *et al.*, 1994).

Environmental Significance of DMSP

Although plants emit many sulfur-containing gases, such as H_2S (hydrogen sulfide), COS (carbonyl sulfide), and SO_2 (sulfur dioxide), the primary biological source of sulfur transferred to the atmosphere is DMS (Steudler and Peterson, 1984; Cooper and Matrai, 1989; Rennenberg, 1991). DMS and its oxidation products (Hatton *et al.*, 1996) have global effects on weather and nutrient cycling (Charlson *et al.*, 1987). In addition to

its important role in the biogeochemical sulfur cycle, oxidation of DMS in the atmosphere generates cloud condensation nuclei and may be a feedback on global climate. However, the effects of biogenic DMS are hard to quantify in light of our poor understanding of the biochemical processes which regulate its formation (Malin and Kirst, 1997).

In addition to being a compatible solute, DMSP is the main biogenic precursor of atmospheric DMS released from the oceans (Aneja and Cooper, 1989; Keller *et al.*, 1989), and is probably one of the precursors of atmospheric DMS coming from land (Steudler and Peterson, 1984; Dacey *et al.*, 1987; Paquet *et al.*, 1994). In marine envrionments, DMS emissions result from the cleavage of algal DMSP via an enzyme-catalyzed elimination reaction that yields DMS and acrylate (Steinke *et al.*, 1996; Taylor and Visscher, 1996). The reaction is catalyzed by endogenous algal DMSP lyases, and by bacterial enzymes that cleave DMSP in decaying algae and sea water (Steinke *et al.*, 1996). Dacey *et al.* (1987) have suggested that DMS emitted from *S. alterniflora* leaves results from breakdown of DMSP by an endogenous enzyme activity, in a manner similar to what is observed in marine algae. Currently, however, there is no strong evidence for such an enzyme activity in higher plants. Additionally, Hanson and Gage (1996) have suggested that decomposition of SMM and other intermediates in the DMSP pathway may also be a major source of DMS from angiosperms.

In a study of European coastal and shelf waters, Turner *et al.* (1989) found a good correlation between DMSP and DMS, and where bloom-forming algal species were monospecific there was a good correlation between biomass and DMS. In general, however, no simple correlation exists between chlorophyll a and DMS concentration

(Cooper and Matrai, 1989; Turner *et al.*, 1989; Keller and Korjeff-Bellows, 1996). One reason is that nutrient availability, developmental stage, salinity, temperature, and other environmental influences may affect the rate of DMSP formation (Morris *et al.*, 1996; Russell and Howard, 1996; Gibson *et al.*, 1996; Karsten *et al.*, 1996). Another reason is that marine and saltmarsh environments generally comprise many plant species, not all of which produce DMSP (Dacey *et al.*, 1987; Keller *et al.*, 1989). One exception may be saltmarshes, however, where DMSP-accumulating species such as *S. alterniflora* (Figure 2) exist as the predominant or, in many cases, the only angiosperm, defining ecological "hot spots" for terrestrial DMS emissions (Steudler and Peterson, 1984; Dacey *et al.*, 1987; Colmer *et al.*, 1996). In marine algae, DMSP decomposition to acrylate and DMS may be a chemical defense against predation (Wolfe *et al.*, 1997). Additionally, DMS and acrylate resulting from the breakdown of DMSP by DMSP lyase appear to play a significant and important role in allowing marine algae to cope with oxidative stress (Sunda *et al.*, 2002). Therefore grazing and other stresses may influence the formaion of DMSP and its conversion to DMS.

Spartina alterniflora is the dominant angiosperm found in the intertidal mud flat regions of the Florida coast (Figure 2), and is one of the most widespread and abundant North American saltmarsh plants (Thompson, 1991). DMSP accumulates to high levels (30 μmol/g fw) in leaves of *S. alterniflora* (Dacey *et al.*, 1987; Otte and Morris, 1994; Colmer *et al.*, 1996), as it does in very few angiosperms (Hanson and Gage, 1996; Paquet *et al.*, 1995; Paquet *et al.*, 1994). Perhaps marshes dominated by *S. alterniflora* could be studied as simple, one-species earth system models.

CHAPTER 3
INSERTIONAL INACTIVATION OF THE METHIONINE *S*-METHYLTRANSFERASE GENE ELIMINATES THE *S*-METHYLMETHIONINE CYCLE AND INCREASES THE METHYLATION RATIO

Chapter Summary

Plants have the unique ability to synthesize *S*-methylmethionine (SMM) from methionine (Met) and *S*-adenosyl-L-Met (AdoMet) via the enzyme Met *S*-methyltransferase (MMT). The SMM can be reconverted to Met by donating a methyl group to homocysteine, and concurrent operation of this reaction and that catalyzed by MMT sets up a futile cycle known as the SMM cycle. The SMM itself is hypothesized to be an essential methyl donor or a necessary transport form of sulfur, while the SMM cycle is hypothesized to stop depletion of Met pools caused by excess AdoMet synthesis, or to govern AdoMet level and hence regulate the AdoMet/*S*-adenosylhomocysteine (AdoHcy) ratio (the methylation ratio). To test these hypotheses, insertional *mmt* mutants of Arabidopsis and maize were isolated and analyzed. Both mutants lacked the capacity to produce SMM and thus had no SMM cycle. Nevertheless, they grew and reproduced normally. Arabidopsis seed sulfur contents were measured and found not to differ. These findings rule out an indispensable role for SMM as a methyl donor or in sulfur transport. The Arabidopsis mutant had significantly higher AdoMet and lower AdoHcy levels than the wild type, however, and consequently a higher methylation ratio (13.8 versus 9.5). Free Met and thiol pools were unaltered in this mutant, although there was a 50% decrease in free threonine (Thr) and changes in other amino acids. These data

indicate that the SMM cycle contributes to regulation of AdoMet levels rather than to preventing depletion of free Met. Since AdoMet is known to activate Thr synthase, that Thr level was not higher but lower in the *mmt* mutant implies that AdoMet is kept away from Thr synthase, an enzyme which is located in the chloroplasts.

Introduction

The synthesis of *S*-methyl-Met (SMM) is a unique feature of the sulfur and one -carbon metabolism of angiosperms and other plants (Pokorny *et al.*, 1970; Mudd and Datko, 1990; Ranocha *et al.*, 2001). The metabolite SMM is formed by the *S*-adenosyl-L -Met (AdoMet)-dependent methylation of Met, catalyzed by Met *S*-methyltransferase (MMT) (Bourgis *et al.*, 1999). The SMM can be reconverted to Met by transferring a methyl group to homocysteine, in a reaction mediated by homocysteine *S*-methyl-transferase (HMT) (Ranocha *et al.*, 2000). The tandem action of MMT and HMT, together with that of AdoMet synthetase and *S*-adenosylhomocysteine (AdoHcy) hydrolase, sets up a futile cycle (the SMM cycle) in which Met is methylated to give SMM, and is then reconverted to Met (Mudd and Datko, 1990). This cycle in effect short-circuits the activated methyl cycle (Figure 4), and each of its turns hydrolyzes a molecule of ATP to adenosine, pyrophosphate, and (ortho)phosphate. Analyses of MMT and HMT expression, and of interconversion of Met and SMM, indicate that the SMM cycle operates throughout the plant (Ranocha *et al.*, 2001). Furthermore, radiotracer kinetic data show that the SMM cycle is a large item in the methyl budget of Arabidopsis leaves, where it consumes half the AdoMet produced (Ranocha *et al.*, 2001).

The functions of SMM and its seemingly wasteful cycle are for the most part unknown. The only established role for SMM is in transporting reduced sulfur in the

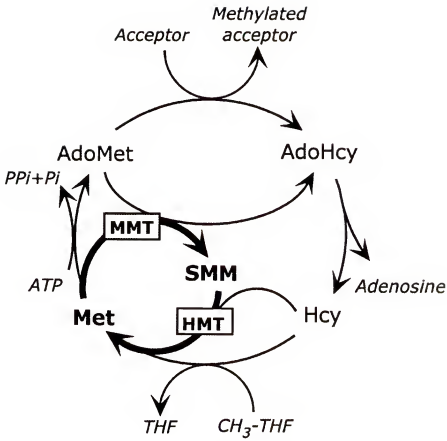

Figure 4. The SMM cycle and its relationship to the activated methyl cycle. The reactions catalyzed by MMT and HMT are in bold. AdoHcy, *S*-adenosyl-homocysteine; CH_3-THF, 5-methyltetrahydrofolate; THF, tetrahydrofolate.

phloem, for which there is qualitative evidence in a range of plants including Arabidopsis (Bourgis *et al.*, 1999). The importance of SMM relative to other translocated forms of sulfur has been quantified only in wheat, where it accounts for half the sulfur moving to developing grains (Bourgis *et al.*, 1999). The contribution of SMM to sulfur transport may be much less in other species (Bourgis *et al.*, 1999), however, and may well vary with developmental stage and sulfur nutrition (Sunarpi and Anderson, 1997; Fitzgerald *et al.*, 2001). A hypothetical role for SMM is as a methyl donor for a plant-specific reaction (Giovanelli *et al.*, 1980). This role has not been tested but would explain why plants alone have SMM.

Roles in transport or methylation might explain why plants produce SMM, but not why there is futile cycling of SMM throughout the plant. Two hypotheses have been advanced to justify this cycling. The first is that the SMM cycle prevents overshoots in AdoMet synthesis from depleting the free Met pool required for protein synthesis (Mudd and Datko, 1990), by providing a way to convert Met moieties locked up in AdoMet back to free Met. The second hypothesis is that the SMM cycle is a means whereby plants control AdoMet level. It is important to note here that in plants there are no feedback loops between AdoMet and the enzymes involved in its synthesis as there are in other eukaryotes (Ranocha *et al.*, 2001; Roje *et al.*, 2002). Furthermore, controlling the levels of AdoMet and AdoHcy is considered crucial to the many methyl transfer reactions that take place in cells: AdoHcy is a potent competitive inhibitor of methyltransferases (Cantoni *et al.*, 1979) so that the AdoMet:AdoHcy ratio (the methylation ratio) determines the activity of these enzymes (Cantoni, 1977).

Computer modeling of the SMM cycle in mature Arabidopsis leaves, based on radiotracer labeling kinetic data and chemical pool size measurements for wild-type plants, favored the second of these two hypotheses (Ranocha *et al.*, 2001). When the SMM cycle was eliminated *in silico*, AdoMet level increased by up to 160% but steady -state free Met levels did not change. Moreover, the free Met pool recovered from a simulated overshoot in AdoMet synthesis almost as fast in the absence of the SMM cycle as in its presence.

In the present study, the function of SMM and its cycle was investigated by isolating and characterizing insertional knockout mutants of MMT in Arabidopsis and maize, which both have single *MMT* genes (Bourgis *et al.*, 1999). It was found that

SMM is in fact dispensable, but that eliminating it results in expansion of the AdoMet pool, as previously predicted by *in silico* modeling, and the increase in AdoMet corresponds to a significant increase in the methylation ratio.

Materials and Methods

Plants and Growing Conditions

Arabidopsis was routinely grown in Fafard Super Fine Germinating Mix (Fafard Inc., Agawam, MA) at 22°C in 12-h days (80-150 μmol photons m^{-2} s^{-1}) and irrigated with deionized water. Arabidopsis plants used for thiol analysis were cultured at 22°C in 16-h days (30 μmol photons m^{-2} s^{-1}) for three weeks on MS solid medium containing 0.8% (w/v) agarose, 1% (w/v) sucrose, and 1.5 mM or 30 μM sulfate. Maize for seed production was grown in the field, or in 34-cm pots of Fafard 3B mix in a naturally lit greenhouse with a minimum temperature of 18°C, and irrigated with general-purpose fertilizers. Maize plants for experiments were grown in the greenhouse or a growth chamber (12-h day, 200-300 μmol photons m^{-2} s^{-1}, 25°C day/21°C night), and were irrigated with tap water containing Masterblend 20-20-20 soluble fertilizer (Masterblend International Inc., Chicago, IL).

Arabidopsis Mutant Isolation

Pools of DNA from a collection of 60,480 Arabidopsis (ecotype WS) lines mutagenized with the pD991 vector were screened by PCR for insertions in the *MMT* locus, using the University of Wisconsin Arabidopsis Gene Knockout Service Facility (http://www.biotech.wisc.edu/Arabidopsis). Because the *MMT* locus is large (6.6 kb), insertions into the 5' and 3' halves of the gene were sought separately, using two pairs of

MMT-specific forward and reverse primers: 5'-CGCTTTTTCTTCTCTATTACTGCA-ATCAC-3' and 5'-TGATCATCTGATTTATCCATGCTAGTGTC-3' for the 5' half, and 5'-CTGATGAGAAGATTCCATTCCTAGCCTAT-3' and 5'-GCGAGTTATTTAGAA-ACAACAGAACAAAG-3' for the 3' half. These primers yield fragments that overlap by 1.2 kb in the center of the gene. To identify and localize T-DNA insertions, these primers were used in conjunction with the T-DNA left-border primer 5'-CATTTTATAA-TAACGCTGCGGACATCTAC-3'. When an insertion was identified, the PCR-amplified product spanning the insertion site was directly sequenced to determine its position. A second, nested left-border primer (5'-TTTCTCCATATTGACCATCATACTCATTG-3') was used to confirm the PCR result and the insert position. The genotypes of plants used for experiments were verified by PCR using DNA from cotyledon samples as template, plus the second and third *MMT*-specific primers above (to identify wild-type alleles), and the second *MMT*-specific primer and the first left-border primer (to identify mutant alleles). The design of *MMT*-specific primers and isolation of *mmt* mutants were carried out by Philippe Ranocha at the University of Florida.

Maize Mutant Isolation

A Pioneer Hi-Bred collection of 42,000 F_1 maize plants mutagenized with Robertson's Mutator (*Mu*) element was screened for *Mu*-containing *MMT* alleles by Mitchell C. Tarczynski, Robert B. Meeley, and Changjiang Li (Pioneer Hi-Bred International). Their efforts were aided by that of Philippe Ranocha (University of Florida). The maize population was screened by PCR as described previously (Bensen *et al.*, 1995). Inserts in the 5' and 3' halves of the gene were sought separately as above; only the 3'-end screening was successful, using the *MMT*-specific forward and reverse

primers: 5'-CAGTACCTT-CAGCAGTGAATGCGTCTGT-3' and 5'-ATTGCTCCA-TCAGGCACCATTCATCTGAG-3'. Insertion site position was determined by cloning and sequencing *MMT*::*Mu* PCR products. The F_2 plants heterozygous for the *mmt* mutation were crossed to inbred line PHN46, followed by two generations of selfing with or without a subsequent outcross to hybrid 46242 x 46243. For use in experiments, homozygous *mmt* mutant and wild-type progeny from ears of selfed heterozygotes were identified by PCR using leaf DNA as template, and the above *MMT*-specific primers together with the *Mu*-specific primer 5'-AGAGAAGCCAACGCCAWCGCCTCYA-TTTCGTC-3'. The outcrossing procedure created greater uniformity, and comparing segregating progeny from the same ears reduced the effects of background mutations.

Southern Analysis

Arabidopsis genomic DNA was isolated from 2-g batches of rosette leaves pooled from 25 plants as described previously by Dellaporta (1994). The isolated DNA (5 μg) was digested, separated by 0.7% (w/v) agarose gel, and transferred to a NitroPure nitrocellulose membrane (Osmonics, Minnetonka, MN). The hybridization was at 65°C in 5x SSC, 5x Denhardt's solution, 1% (w/v) SDS, and 1 mM EDTA. The probe was the PCR-amplified T-DNA insert of plasmid pD991 (Campisi *et al.*, 1999), labeled with ^{32}P by random hexamer priming. The final wash was at 65°C in 0.1x SSC containing 0.1% (w/v) SDS.

Arabidopsis Growth Measurements

Arabidopsis plants were grown (one per 3.5-cm pot) in the conditions described above. Heights of ten plants were measured daily. Each week ten shoots were harvested and weighed immediately (fresh), and then weighed again after lyophilization. For the

final harvest, shoots were dried at 70°C for 12 h, weighed, and threshed. The separated seeds were then weighed.

***In-Vivo* Measurement of MMT Activity**

For Arabidopsis, sets of three mature rosette leaves from 3.5-week old plants were given a pulse of L-[^{35}S]Met (0.34 μCi, 40 pmol per leaf) for 90 min at 24 ± 1°C in the light as described previously (Ranocha *et al.*, 2001). For maize, 25-mm sections (weighing about 50 mg) were cut from the mid-blade position of the youngest fully -expanded leaf of plants at the five-leaf stage. The midrib was shaved from the abaxial surface of each section and a 1.0-μCi (130 pmol) dose of L-[^{35}S]Met dissolved in 3.3 μL of water was applied to the cut surface. After the labeled droplet was absorbed, 8 μL of water was added to wash the label into the tissue, and sections were incubated on moist filter paper for 2 h as above. After incubation, Arabidopsis and maize samples were processed as described by Ranocha *et al.* (2001) to measure [^{35}S]SMM formation, as well as L-[^{35}S]Met uptake and incorporation into protein. The [^{35}S]SMM was analyzed using TLC system 1 and thin-layer electrophoresis (TLE) systems 1 and 2 described by James *et al.* (1995b), and by its decomposition upon treatment with 2 N NH_4OH at 95-100°C for 3 h (Macnicol, 1986).

SMM Determination

Arabidopsis samples obtained by pooling the shoots (rosettes) of five plants, and maize leaf samples from individual plants with five to six leaves, were frozen in liquid N_2 and stored at -80°C until analysis. The SMM content of each sample was determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis of cation fractions, using an internal standard of [$^2H_6$]SMM (Kocsis *et al.*, 1998). The MS

analyses were carried out by Douglas A. Gage and Eric S. Simon at Michigan State University.

AdoMet and AdoHcy Determination

The AdoMet and AdoHcy were quantified by HPLC (high-performance liquid chromatography), as described previously (Capdevila and Wagner, 1998; Moffatt *et al.*, 2002). Arabidopsis leaf samples (150-200 mg fresh weight) were extracted in one volume of 20% (w/v) trichloroacetic acid (TCA) and clarified by centrifugation. After removal of TCA, the supernatant was separated on a C-8 column, followed by conversion of AdoMet and AdoHcy to fluorescent isoindoles by derivatization with naphthalene-dialdehyde and cyanide. The fluorescent derivatives were measured during isocratic elution from a C-18 column (Capdevila and Wagner, 1998). The AdoMet and AdoHcy peaks were identified and quantified by reference to authentic standards. Data were corrected for recovery using AdoMet and AdoHcy spikes of 16 and 1.2 nmol, respectively, added to representative samples. The AdoMet recovery was 63% and AdoHcy recovery was 24%. The HPLC analyses were carried out on prepared samples by Conrad Wagner at Vanderbilt University.

Amino Acid Analysis

Samples of Arabidopsis rosette leaves pooled from five plants (0.4-0.5 g fresh weight) were frozen in liquid N_2 immediately after harvest and stored at -20°C until analysis. The samples were weighed exactly and extracted with 10 mL methanol for 48 h at 4°C in darkness; each was spiked with 250 nmol α-amino-*n*-butyrate as internal standard, plus 100 nmol [$^{13}C_5$]Met for Met quantification by isotope dilution MS. The methanol extract was phase-separated by adding 5 mL chloroform plus 6 mL water, and the aqueous phase was dried in an air stream. Dried aqueous phases were redissolved in

1 mL water and applied to 4.5 cm x 1 cm columns of Dowex-1 [OH^-]; after washing columns with water, amino acids were eluted with 6 mL 2.5 N HCl and dried as above. The dried eluates were redissolved in 1 mL water and applied to 4.5 cm x 1 cm columns of Dowex-50 [H^+], which were washed with water and eluted with 6 mL 6 N NH_4OH. After drying, eluates were derivatized to *N*-(*O*,*S*)-heptafluorobutyryl isobutyl esters by reaction with isobutanol:acetyl chloride (5:1, v/v; 120°C, 20 min), followed by heptafluorobutyric anhydride (120°C, 10 min) (Rhodes *et al.*, 1986). After evaporating excess heptafluorobutyric anhydride, samples were redissolved in 200 μL of ethyl acetate:acetic anhydride (1:1, v/v) for analysis by GC (gas chromatography) and by electron ionization GC-MS as described previously (Rhodes *et al.*, 1986), with the exception that a Finnigan GCQ mass spectrometer was used. Amino acid analyses of samples were carried out by David Rhodes and Gregory J. Peel at Purdue University.

Thiol Analysis

Samples comprised the rosette leaves or roots from two or three pooled Arabidopsis plants. Thiols were determined as monobromobimane derivatives by HPLC with fluorescence detection (Fahey and Newton, 1987). Tissues were extracted using 5 volumes of 0.01 N HCl for leaves, and 10 volumes for roots. Three μL of 5 mM DTT and 50 μL of CHES buffer (100 mM, pH 9.3) were added to 20-μL portions of extracts, and the mixtures were incubated at 37°C for 20 min to allow reduction to occur. Five μL of 30 mM monobromobimane were then added, and incubation at 37°C was continued for 45 min. The derivatization reaction was stopped by adding 30 μL of glacial acetic acid, and the volume was brought to 160 μL with water. Aliquots (20 μL) were analyzed by HPLC, using a Mightysil RP-18GP (5 μm) 250 x 4.6 mm column (Kanto, Portland, OR) eluted from 0 to 15 min and from 22 to 30 min with 99% buffer A + 1% buffer B (v/v),

and from 15 to 22 min with buffer B. Buffer A was 8% methanol:92% water (v/v) containing 0.25% (w/v) acetic acid (pH 3.9); buffer B was 90% methanol:10% water (v/v) containing 0.25% (w/v) acetic acid (pH 3.9). The flow rate was 1.2 mL min^{-1}. To detect the monobromobimane derivatives, the fluorescence of the column effluent was monitored at an excitation wavelength of 390 nm and an emission wavelength of 482 nm. Thiol analysis was conducted at Chiba University, Japan by Kazuki Saito and Motoko Awazuhara on plants grown from seeds that were obtained from self-pollinated plants screened and shown to be homozygous mutant or wild type.

Determination of Sulfur Content of Seeds

Arabidopsis seed samples (100 mg) were extracted by treatment with 4 mL of HNO_3 (65% w/w) and 3 mL of H_2O_2 (30% w/w) in high-pressure Teflon vessels, in which samples were incubated in a microwave oven for 20 min at 195°C and 1.2 MPa. After cooling, 50 mL of bidistilled water were added and extracts were transferred to polyethylene bottles. The extracts were diluted (1:4) with 7% (w/w) HNO_3, and their sulfur contents were determined by inductively coupled plasma optical emission spectroscopy using a Varian Liberty 150 instrument at a wavelength of 182.034 nm. Sulfur measurement was carried out by Stefan Mellema at the University of Berne, Switzerland on seeds from self-pollinated plants that were PCR screened and shown to be homozygous mutant or wild type.

Results

Isolation of Arabidopsis and Maize *mmt* Insertional Mutants

An Arabidopsis *mmt* mutant was isolated by PCR screening a T-DNA insertion population (Krysan *et al.*, 1999), and a homozygous line was selected for study.

Sequencing of genomic DNA showed that the insertion was located close to the 3' end of intron 7 (Figure 5A). An insertion at this point is expected to stop formation of a competent enzyme, given the large size of the T-DNA (Campisi *et al.*, 1999). Southern blot analysis of plants homozygous for the T-DNA-tagged *mmt* allele and their wild-type siblings demonstrated that only the former harbored inserts, indicating that the T-DNA is inserted only at the *MMT* locus (Figure 6). All further experiments with Arabidopsis were carried out with these two sibling populations or their self-pollinated progeny.

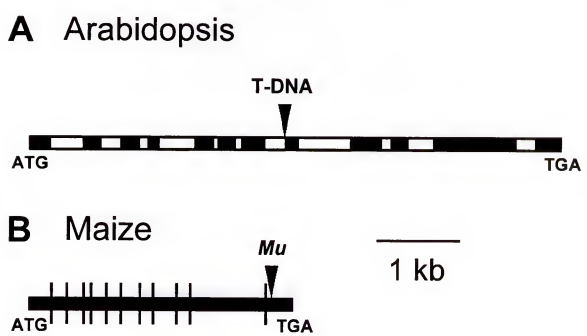

Figure 5. Insertion sites in MMT sequences.
A, The Arabidopsis *MMT* locus showing the T-DNA insertion in intron 7; black boxes represent exons and white boxes introns. B, The maize *MMT* cDNA showing the site of the *Mu* insertion near the 3' end of the coding sequence. Intron positions predicted from alignments to Arabidopsis and rice genomic sequences are shown by vertical lines.

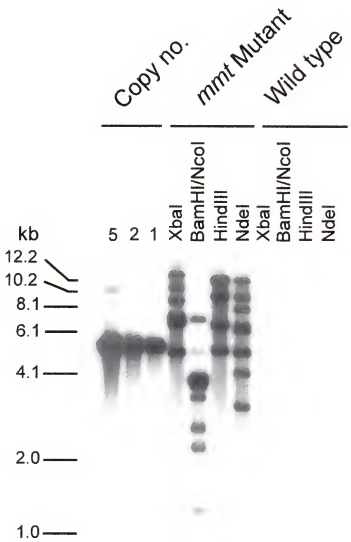

Figure 6. Southern blot analysis of T-DNA-containing sequences in *mmt* mutant and wild-type Arabidopsis plants.
Genomic DNA was extracted from plants shown by PCR to be homozygous for the mutant *mmt* allele or the wild-type *MMT* allele. The DNA was digested with the enzymes indicated. Blots were probed with the whole T-DNA region from the pD991 vector, containing the GUS and kanamycin resistance genes (Campisi *et al.*, 1999). Genomic reconstructions were made with linearized pD991 DNA equivalent to one, two, and five copies per haploid genome; the results are consistent with insertion of a small number of copies of the T-DNA at a single locus in the *mmt* mutant. The positions of DNA size markers are indicated on the left.

An analogous strategy was used to screen a population of maize plants mutagenized by Robertson's Mutator (*Mu*) element (Bensen *et al.*, 1995; Meeley and Briggs,

1995). This procedure identified mutants harboring a *Mu* element inserted after the codon specifying amino acid 989 of the 1091-residue protein (Figure 5B). An MMT protein truncated at this point is unlikely to be functional since it would lack a putative ligand binding region (Bourgis *et al.*, 1999). Because plants from the *Mu*-mutagenized population that was screened typically harbor many *Mu* elements and have low vigor, mutants were outcrossed once or twice to vigorous genotypes. Progeny heterozygous for the mutant *mmt* allele were identified by PCR and selfed; the progeny were screened by PCR to identify homozygous mutant and wild-type individuals, and these were used for experiments.

SMM Levels and MMT Activities

As a first and direct test of the metabolic phenotype of the *mmt* mutants, leaf SMM levels were determined by MALDI-MS analysis of a cationic fraction from which inorganic salts had been removed. An internal standard of [*methyl*-$^2H_6$]SMM was used for quantification. This procedure gave spectra with strong signals for endogenous SMM and the standard, and no other significant peaks (Figure. 7). In both Arabidopsis and maize, the level of SMM in *mmt* mutant homozygotes was below the detection limit, which was 1-2% of the level in the corresponding wild type (Table I). To corroborate this result, MMT activities were measured *in vivo* by supplying leaves with tracer doses of [^{35}S]Met and measuring ^{35}S incorporation into SMM (Table II). No activity was detected in Arabidopsis mutants, and very little in maize (Table II). The trace of [^{35}S]SMM synthesis seen in maize mutants (0.8% of the wild type) could be due to vestigial activity in the truncated MMT protein resulting from *Mu* element insertion (Figure 5B), or to a secondary activity of another methyltransferase (Katz and Gerhardt, 1990). In any case, the data of Table II confirm that these insertional mutants of

Arabidopsis and maize are in effect MMT knockouts. Table II also shows that [^{35}S]Met incorporation into protein was normal in *mmt* mutants, which suggests that the endogenous metabolic pool of free Met is unaltered. A larger free Met pool would reduce labeled protein synthesis via isotope dilution, and a smaller pool would increase it.

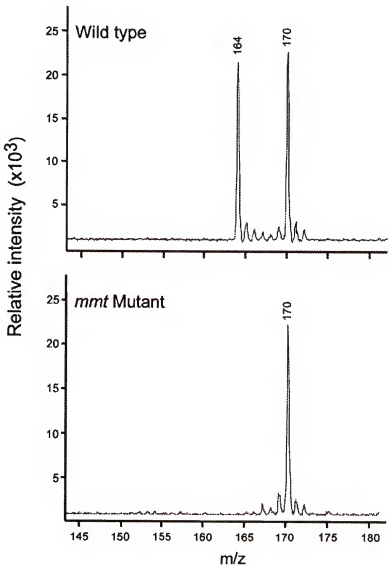

Figure 7. Analysis by MALDI-MS of the cation fractions from leaves of wild-type and *mmt* mutant maize.
The peaks at *m/z* 164 and 170 correspond to endogenous SMM and the [*methyl*-$^2H_6$]-SMM internal standard, respectively. The spectra shown are for representative samples.

Table I. The SMM levels in leaves of wild-type and *mmt* mutant Arabidopsis and maize. The amount of SMM was determined by MALDI-MS in samples (approximately 0.5 g fresh weight) comprising pooled rosette leaves from five Arabidopsis plants harvested just before bolting, or leaves from individual maize plants at the five- to six-leaf stage. Data are means of three (Arabidopsis) or four (maize) replicates ± SE. FW, fresh weight. The MS analyses were carried out by Douglas A. Gage and Eric S. Simon at Michigan State University.

Arabidopsis		Maize	
Wild-type	*mmt* Mutant	Wild-type	*mmt* Mutant
		nmol g^{-1} *FW*	
41 ± 5	<1	105 ± 27	<1

Growth Characteristics and Seed Sulfur Contents

Despite the loss of SMM, neither Arabidopsis nor maize mutants appeared to differ from wild-type plants in morphology or fertility (Figure 8). This was verified for Arabidopsis by measuring the growth rates, flowering dates, and seed yields of mutant and wild-type homozygotes. Throughout growth, there were no significant differences in height or dry weight (Figure 9), or in fresh weight (see APPENDIX). The flowering dates of mutant and wild type coincided (Figure 9). Additionally, the total weight of seeds per plant and seed size did not differ significantly. The wild type and mutant produced 144 ±15 and 138 ±15 mg seeds per plant (mean ± SE, $n = 20$), with average seed weights of 16.2 and 17.1 µg, respectively.

Table II. *In-vivo* conversion of tracer amounts of [^{35}S]Met to [^{35}S]SMM by leaf tissues of wild-type and *mmt* mutant Arabidopsis and maize.
Sets of three Arabidopsis leaves or single 25-mm maize leaf segments were supplied with 1.0 μCi (120-130 pmol) of [^{35}S]Met for 1.5 or 2 h in the light, then washed in 0.1 mM Met for 0.5 h to remove unabsorbed label. This [^{35}S]Met dose is very small compared to endogenous free Met content (Ranocha *et al.*, 2001). At least 80% of the [^{35}S]Met was absorbed. Data are expressed as nCi per three leaves (Arabidopsis) or per segment (maize) and are means of three replicates ± SE.

Genotype	[^{35}S]SMM	[^{35}S]Protein	[^{35}S]Met remaining
		nCi	
Arabidopsis wild-type	50 ± 8	132 ± 13	142 ± 4
Arabidopsis *mmt* mutant	<0.4[a]	112 ± 17	154 ± 7
Maize wild-type	84 ± 26	147 ± 27	340 ± 26
Maize *mmt* mutant	0.8 ± 0.2[b]	150 ± 10	437 ± 15

[a]The detection limit was 0.4 nCi.
[b]The identity of the trace of [^{35}S]SMM in *mmt* mutant maize was confirmed by co-migration with authentic SMM in three thin-layer separatory systems and by decomposition in hot base.

In view of the potential role of SMM in long-distance sulfur transport (Bourgis *et al.*, 1999), the sulfur contents of wild-type and mutant Arabidopsis seeds were measured. The values obtained were not significantly different: 8.08 ±0.17 mg g^{-1} for the wild type and 8.02 ± 0.12 mg g^{-1} for the mutant (means ±SE for six replicates). This suggests that loss of SMM reduced neither protein nor glucosinolate accumulation in seeds, the sulfur in Arabidopsis seeds being about equally divided between these categories (Haughn *et al.*, 1991). Consistent with there being no difference in seed sulfur reserves, wild-type

and mutant seed germination rates and seedling vigor were the same when grown or cultured under the conditions described above (see APPENDIX).

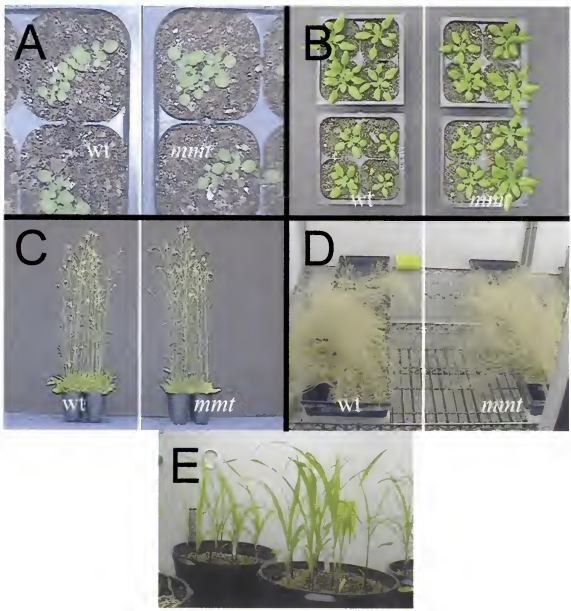

Figure 8. Based on observations of morphology and fertility, there are no apparent differences in growth and development of *mmt* mutant versus wild-type Arabidopsis or maize.
Arabidopsis at A, early rosette stage; B, late rosette stage; C, just after bolting and anthesis; and D, after ripening. Homozygous mutant, homozygous wild -type, and heterozygous maize seedlings growing together are indistinguishable (E). wt, wild type. *mmt*, mutant.

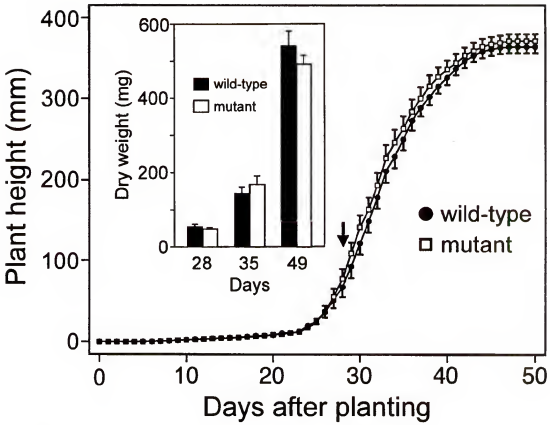

Figure 9. Growth of wild-type and *mmt* mutant Arabidopsis plants. Heights of ten individuals were measured daily in each of two experiments; dry weights (inset) were measured weekly for ten individuals. Data are means ± SE. The flowering date (arrow) did not differ significantly between wild -type and mutant plants (28.9 ± 0.4 and 28.5 ± 0.4 days after planting, respectively).

AdoMet and AdoHcy Contents

Because SMM synthesis is a major fate of AdoMet in mature Arabidopsis leaves (Ranocha *et al.*, 2001), the levels of AdoMet and AdoHcy in wild-type and mutant leaves was measured by HPLC-fluorescence analysis of isoindole derivatives (Capdevila and Wagner, 1998). The AdoMet level was significantly higher in the mutant, and AdoHcy level significantly lower, so that the methylation ratio increased from 9.5 to 13.8 (Table III). The AdoMet and AdoHcy levels that were observed here in wild-type leaves are comparable to those recently reported for Arabidopsis by Moffatt *et al.* (2002).

Table III. The AdoMet and AdoHcy levels in leaves of wild-type and *mmt* mutant Arabidopsis.
The AdoMet and AdoHcy were determined as their isoindole derivatives by fluorescence-HPLC using samples (0.15 – 0.2 g fresh weight) drawn from pooled rosette leaves harvested from five plants just prior to bolting. Data are means of four replicates ± SE and have been corrected for recovery. Differences between wild type and mutant that are significant at $P = 0.05$, 0.01, or 0.001 are indicated by one, two, or three asterisks, respectively. The HPLC analyses were carried out on prepared samples by Conrad Wagner at Vanderbilt University.

Genotype	AdoMet	AdoHcy	Methylation Ratio (AdoMet/AdoHcy)
	nmol g^{-1} *FW*		
Wild type	13.0 ± 0.4	1.37 ± 0.06	9.5 ± 0.4
mmt Mutant	14.9 ± 0.8*	1.09 ± 0.06**	13.8 ± 0.5***

Amino Acid and Thiol Contents

The free amino acid contents of Arabidopsis rosette leaves were determined by GC and GC-MS to assess the impact of eliminating SMM on amino acid metabolism as a whole. The contents of all twelve amino acids that were detected were either unaltered or modestly reduced, so that the total amino acid content of mutant leaves was 38% lower than wild type (Table IV). Two amino acids, Met and Thr, were of special interest because: (a) SMM is synthesized from Met, and (b) Thr synthesis competes with Met (and SMM) synthesis for a common precursor, and may be regulated by AdoMet (Galili and Höfgen, 2002). There was no change in Met content, in agreement with the prediction based on radiolabeling data (see above). The Thr content was 50% lower in the mutant, but this was not a specific effect since other amino acids showed similar decreases (Table IV).

Table IV. Free amino acid levels in leaves of wild-type and *mmt* mutant Arabidopsis plants.
Amino acids were determined using pooled rosette leaves (0.4 – 0.5 g) from five plants harvested just before bolting. Data are means of three replicates ± SE. The Thr and Met values are in bold for emphasis. Amino acid analyses of samples were carried out by David Rhodes and Gregory J. Peel at Purdue University, and this table comprises part of the paper published by Kocsis *et al.* (2003).

Amino acid	Amino acid level		RATIO
	Wild-type	*mmt* Mutant	*mmt* Mutant/ Wild-Type
		nmol g^{-1} FW	
Met	**28 ± 1**	**31 ± 5**	**1.11 ± 0.22**
Thr	**361 ± 17**	**179 ± 21**	**0.50 ± 0.08**
Ala	518 ± 46	522 ± 178	1.01 ± 0.43
Gly	84 ± 16	61 ± 14	0.73 ± 0.31
Val	71 ± 3	49 ± 6	0.69 ± 0.11
Ser	974 ± 89	415 ± 20	0.43 ± 0.06
Leu	27 ± 1	23 ± 4	0.85 ± 0.18
Ile	26 ± 1	21 ± 3	0.81 ± 0.15
Pro	236 ± 66	124 ± 14	0.53 ± 0.21
Asx[a]	709 ± 14	463 ± 36	0.65 ± 0.06
Phe	21 ± 1	14 ± 2	0.67 ± 0.13
Glx[b]	1680 ± 36	1021 ± 87	0.61 ± 0.06
Total	4735 ± 290	2923 ± 389	0.62 ± 0.12

[a]Asp + Asn
[b]Glu + Gln

Perturbations of sulfur metabolism were sought by determining Cys and glutathione levels in Arabidopsis leaves and roots. The leaves and roots of mutant plants did not differ in their levels of Cys or glutathione from the corresponding wild-type organs when sulfate supply was adequate (1.5 mM), or when the sulfate concentration in the medium was reduced to 30 μM (Table V).

Table V. Thiol levels in leaves and roots of wild-type and *mmt* mutant Arabidopsis plants.
Leaves and roots were harvested from three-week old homozygous wild-type and mutant plants cultured on medium containing 1.5 mM or 30 μM sulfate. Data are means of five replicates ± SE. None of the differences between wild -type and mutant plants are significant at $P = 0.05$. Thiol analysis was conducted at Chiba University, Japan by Kazuki Saito and Motoko Awazuhara on plants grown from seeds that were obtained from self-pollinated plants screened and shown to be homozygous mutant or wild type.

Sulfate level	Thiol	Leaves		Roots	
		Wild-type	*mmt* Mutant	Wild-type	*mmt* Mutant
			nmol g^{-1} *FW*		
1.5 mM	Cysteine	34 ± 6	33 ± 3	41 ± 4	39 ± 4
	Glutathione	492 ± 52	483 ± 46	179 ± 34	180 ± 19
30 μM	Cysteine	9 ± 1	10 ± 1	31 ± 12	21 ± 3
	Glutathione	120 ± 19	101 ± 46	100 ± 19	75 ± 15

Discussion

The results show that eliminating MMT, and hence SMM, has no appreciable effect on growth or development in normal culture conditions. The same was recently

reported for an independent Arabidopsis *mmt* knockout line that was isolated to study selenium volatilization (Tagmount *et al.*, 2002), although SMM levels and growth were not quantified. It is therefore unlikely that SMM is a methyl donor for any essential reaction. This long-standing hypothetical role for SMM (Giovanelli *et al.*, 1980) can thus be struck off the list of possibilities, at least for plants growing in favorable environments. Likewise, the normal pattern of growth, thiol levels, and seed sulfur contents in *mmt* mutants argue against an essential role for SMM in long-distance sulfur transport. Since SMM occurs in Arabidopsis and maize phloem (Bourgis *et al.*, 1999), the lack of impact of eliminating SMM suggests that other sulfur sources such as glutathione or sulfate can readily replace it, or perhaps that SMM is normally only a minor form of phloem sulfur in these plants.

The data also permit discrimination between the alternative hypothetical functions of the SMM cycle, namely that it forestalls depletion of the free Met pool due to overshoots in AdoMet synthesis (Mudd and Datko, 1990), or that it controls AdoMet levels (Ranocha *et al.*, 2001). The first hypothesis predicts that the metabolic pool of free Met will fluctuate in MMT knockouts, to the detriment of protein synthesis. While fluctuations would presumably be damped by the relatively constant growth conditions used, it is improbable that they would disappear, above all in meristematic regions where the metabolic Met pool turns over very fast and there is little or no vacuolar storage pool of Met to act as a reserve (Mudd and Datko, 1990; Ranocha *et al.*, 2001). The lack of growth defects in the *mmt* mutants therefore argues against this hypothesis. That the free Met content in mutant Arabidopsis leaves is normal likewise argues against it.

In contrast, the second hypothesis about the function of the SMM cycle receives substantial support from the data. This hypothesis predicts a rise in AdoMet level but not Met level in mutant leaves, which is what is observed. It should be noted that the AdoMet accumulation observed in the leaves of *mmt* knockouts is much smaller (15% versus 160%) than predicted by *in silico* modeling of the effect of ablating the SMM cycle (Ranocha *et al.*, 2001). The model used was designed to predict short-term metabolic responses, however, and did not provide for long-term changes in gene expression, and hence enzyme level, in response to loss of SMM. Such long-term changes are likely *a priori*, and many alterations in transcript levels have in fact been seen in pilot DNA array hybridization experiments with wild-type and *mmt* mutant Arabidopsis (Ranocha and Hanson, unpublished data). Compensatory changes in the expression of genes involved in AdoMet production or consumption may therefore mitigate the impact of the *mmt* mutation. In any event, coupled with the modest opposing change in AdoHcy, the rise in AdoMet level increases the methylation ratio in *mmt* mutant leaves by 45%. Proportional changes of this scale in animal tissues are associated with altered DNA methyltransferase activity and gene expression (Caudill *et al.*, 2001; Halsted *et al.*, 2002; Pascale *et al.*, 2002). It remains to be established whether this is the case in plants.

Raamsdonk *et al.* (2001) have predicted that mutations that are silent when scored on the basis of growth rate should produce obvious effects on metabolite concentrations. This has a firm basis in metabolic control theory because concentration control coefficients (C^S_E), which relate changes in metabolite concentrations S to changes in enzyme levels E, can have large positive or negative values (Fell, 1997). The results seen here

with *mmt* knockout mutants conform to this pattern: the mutants look normal but have a definite metabolic phenotype. This is in principle encouraging for metabolic profiling efforts which, it is hoped, will reveal the phenotype of many silent mutations (Raamsdonk *et al.*, 2001; Trethewey, 2001). It is sobering to note, however, that the GC-MS profile of free amino acids in the Arabidopsis *mmt* mutant was uninformative, and that a clear metabolic phenotype was only evident by analyzing SMM. In high-throughput metabolic profiling projects, amino acids are typically analyzed by GC-MS (Trethewey, 2001) but SMM is not, and cannot be since it is non-volatile. The phenotype of *mmt* mutants would thus be missed by GC-MS profiling technology.

The decrease in Thr level in Arabidopsis *mmt* mutants is noteworthy because a modest increase would in the simplest case be expected. The current view of regulation in the aspartate amino acid family is that the level of AdoMet controls flux partitioning at the branch point between Thr and Met synthesis by activating Thr synthase, a chloroplastic enzyme that competes with cystathionine γ-synthase for the common intermediate *O*-phosphohomoserine (Giovanelli *et al.*, 1989; Galili and Höfgen, 2002). This view stems from the strong allosteric activation of Thr synthase by AdoMet observed *in vitro* (Curien *et al.*, 1998; Laber *et al.*, 1999), but the role of AdoMet in regulating flux to Thr *in vivo* is unclear and there are some contrary findings (Galili and Höfgen, 2002). For example, Arabidopsis *mto3* mutants have lower AdoMet levels but higher Thr levels, and *mto1* mutants that overproduce AdoMet, and transgenics that presumably do so, have normal Thr levels (Inaba *et al.*, 1994; Kim *et al.*, 2002; Shen *et al.*, 2002). That *mmt* mutants have a higher level of AdoMet but a lower level of Thr is thus another instance of the lack of an *in-vivo* relationship between AdoMet level and Thr synthase activation.

One way to reconcile this is to suppose that AdoMet is in part sequestered away from the site of Thr synthase in the chloroplast, and in this connection it is interesting to note that yeast cells sequester AdoMet in the vacuole (Schlenk, 1965).

CHAPTER 4
DIMETHYLSULFONIOPROPIONATE BIOSYNTHESIS IN *SPARTINA ALTERNIFLORA*

Chapter Summary

The osmoprotectant 3-dimethylsulfoniopropionate (DMSP) occurs in Gramineae and Compositae, but its synthesis has been studied only in the latter. The DMSP synthesis pathway was therefore investigated in the saltmarsh grass *Spartina alterniflora* Loisel. Leaf tissue metabolized supplied [^{35}S]methionine (Met) to *S*-methyl-L-Met (SMM), 3-dimethylsulfoniopropylamine (DMSP-amine), and DMSP. The ^{35}S-labeling kinetics of SMM and DMSP-amine indicated that they were intermediates and, consistent with this, the dimethylsulfonium moiety of SMM was shown by stable isotope labeling to be incorporated as a unit into DMSP. The identity of DMSP-amine, a novel natural product, was confirmed by chemical and mass-spectral methods. The *S. alterniflora* readily converted supplied [^{35}S]SMM to DMSP-amine and DMSP, and also readily converted supplied [^{35}S]DMSP-amine to DMSP; grasses that lack DMSP did neither. A small amount of label was detected in 3-dimethylsulfoniopropionaldehyde (DMSP-ald) when [^{35}S]SMM or [^{35}S]DMSP-amine was given. These results are consistent with the operation of the pathway Met → SMM → DMSP-amine → DMSP-ald → DMSP, which differs from that found in Compositae by the presence of a free DMSP-amine intermediate. This dissimilarity suggests that DMSP synthesis evolved independently in Gramineae and Compositae.

Introduction

Dimethylsulfoniopropionate (DMSP) is a sulfonium betaine accumulated by many marine algae (Blunden and Gordon, 1986; Keller *et al.*, 1989) and by certain angiosperms from the families Compositae and Gramineae. The best studied of these are the coastal strand plant *Wollastonia biflora* (L.) DC. (Compositae) and the saltmarsh grass *Spartina alterniflora* Loisel. (Gramineae) (Storey *et al.*, 1993; Colmer *et al.*, 1996). Dimethylsulfoniopropionate is environmentally important as the main biogenic precursor of atmospheric dimethylsulfide (DMS), which has roles in the biogeochemical sulfur cycle, in cloud formation, and in acid precipitation (Malin, 1996). While oceanic DMS fluxes are globally the largest source of atmospheric DMS, those from saltmarshes dominated by *S. alterniflora* are one to two orders of magnitude higher per unit area and may significantly impact atmospheric sulfur budgets on a regional or local scale (Steudler and Peterson, 1984; Aneja and Cooper, 1989).

Dimethylsulfoniopropionate is also physiologically important. Like its analog, glycine betaine, DMSP functions as a compatible solute for enzymes *in vitro* (Gröne and Kirst, 1991; Nishiguchi and Somero, 1992) and as an osmoprotectant for bacteria (Mason and Blunden, 1989; Paquet *et al.*, 1994). Consistent with its having such functions in plants, DMSP has been shown to accumulate to high levels ($\geq$ 100 mM) in the cytoplasm of algal cells (Dickson *et al.*, 1980; Dickson and Kirst, 1986) and in *W. biflora* chloroplasts (Trossat *et al.*, 1998). There is also evidence that DMSP is an effective cryoprotectant (Karsten *et al.*, 1996).

The biosynthesis of DMSP from Met has been partially elucidated in the Compositae *W. biflora* (Hanson *et al.*, 1994b; James *et al.*, 1995b) and *Ratibida pinnata*

(Vent.) Barnhart (Paquet *et al.*, 1995), and its subcellular compartmentation established (Trossat *et al.*, 1996). The only known intermediates in this pathway are *S*-methyl-L-methionine (SMM) and 3-dimethylsulfoniopropionaldehyde (DMSP-ald) (Figure 10). Although conversion of SMM to DMSP-ald entails loss of both the amino and carboxyl groups, there is no evidence that this occurs via DMSP-amine or any other stable intermediate, and ^{15}N-labeling data support a mechanism in which SMM is transaminated and decarboxylated by the same enzyme or by a transaminase-decarboxylase complex (Rhodes *et al.*, 1997). Most, if not all, angiosperms synthesize SMM (Giovanelli *et al.*, 1980; Bezzubov and Gessler, 1992), and many have dehydrogenases that can mediate oxidation of DMSP-ald to DMSP (Trossat *et al.*, 1997; Vojtěchová *et al.*, 1997). It is therefore the conversion of SMM to DMSP-ald that is special to DMSP synthesis.

The DMSP synthesis pathway has also been studied in the marine macroalga *Enteromorpha intestinalis* (L.) Link and in three microalgae (Gage *et al.*, 1997; Summers *et al.*, 1998). It proceeds via the intermediates 4-methylthio-2-oxobutyrate (MTOB), 4-methylthio-2-hydroxybutyrate (MTHB), and 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB), and therefore has no steps in common with the Compositae pathway (Figure 10). This dissimilarity shows that DMSP synthesis evolved independently in algae and in the ancestors of Compositae. It also suggests that the pathway in Gramineae might differ from that in Compositae because these families stand far apart phylogenetically, their progenitors having diverged more than 100 million years ago (Crane *et al.*, 1995). This prompted the current examination of the DMSP synthesis route in *S. alterniflora*. Here, it was found that it is not the same as either the algal or the Compositae pathway. While

resembling the latter in having SMM and DMSP-ald intermediates, it differs in the key SMM → DMSP-ald step.

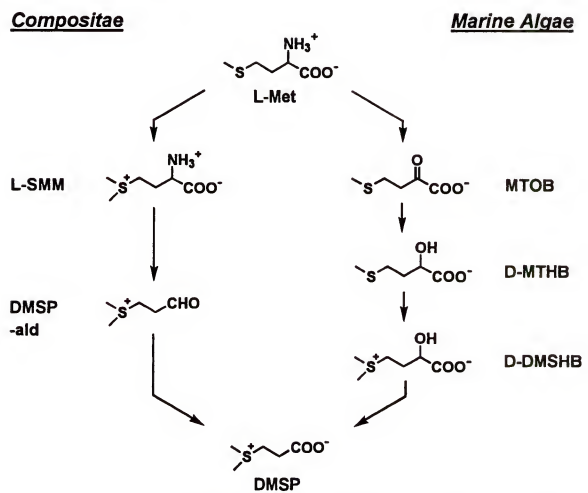

Figure 10. Known steps in DMSP biosynthesis in *W. biflora* (Compositae) and in the marine alga *E. intestinalis*.
The enantiomers of chiral compounds are indicated. No intermediate between SMM and DMSP-ald has been identified in *W. biflora* (James *et al.*, 1995b).

Materials and Methods

Plant Material

Spartina alterniflora Loisel. and *S. patens* (Ait.) Muhl. were collected in Florida from coastal marshes in Crescent Beach and Archie Creek (east Tampa Bay),

respectively. Blocks of soil (3 to 5 dm^3) containing plants were dug up and transferred to undrained plastic containers. The plants were then maintained for up to four months in a naturally-lit greenhouse (minimum temperature 18°C) with the water table close to the soil surface to replace water lost by evapotranspiration; this kept the salinity close to that of the collection site. Since high nitrogen supply decreases DMSP levels (Colmer *et al.*, 1996), no fertilization was given. Other species were fertilized and not salinized: wheat (*Triticum aestivum* L. cv Florida 310), maize (*Zea mays* L. cv NK 508), *Cortaderia selloana* Aschers. & Graebn., and *Oplismenus compositus* Beauv. were greenhouse grown; *Pennisetum purpureum* Schumach PI 300086 was grown in a shade house; and *Cynodon dactylon* Pers. cv Floratex and *Bambusa glaucescens* (Willd.) Sieb. ex Munro cv Stripestem were field grown. The DMSP was assayed in leaf samples (30 mg fresh weight) by a GC method (Paquet *et al.*, 1994). Autumn fern (*Dryopteris erythrosora* [Eaton] Kuntze) was purchased locally and its fronds used to prepare an acetone powder with Met decarboxylase activity (Stevenson *et al.*, 1990).

Chemicals

The iodide form of SMM (Sigma) was converted to the HCl form by adsorption to Dowex-50 (H^+) and elution with 2.5 N HCl; it was lyophilized and neutralized before use with one equivalent of $KHCO_3$. The DMSP-amine chloride was prepared from 1 mmol of 3-methylthiopropylamine (MTP-amine; Chem Service, West Chester, PA) by neutralizing with HCl and treating with 0.7 mL of 6 N HCl containing 2 mmol of MeOH at 110°C for 4 h (Lavine *et al.*, 1954). The product (yield = 75%) was isolated using Dowex-50 as described above and lyophilized; purity was $\geq$ 98%, as determined by TLC and TLE. The iodide form of DMSP-ald was synthesized as described previously (James

et al., 1995b). The DMSP hydrochloride was obtained from Research Plus, Inc. (Bayonne, NJ).

Labeled Compounds

To give the desired specific activity, [^{35}S]Met (44 GBq μmol^{-1}, NEN-DuPont) was mixed with unlabeled Met (Sigma). For experiments with leaf tissue, it was then treated with Dowex-1 (formate) and Dowex-50 (NH_4^+) to remove acidic and basic impurities, respectively. The [^{35}S]SMM (370 kBq nmol^{-1}) was synthesized by chemical methylation of [^{35}S]Met as described by Gage *et al.* (1997). To prepare the [^{35}S]DMSP -amine (165 kBq nmol^{-1}), [^{35}S]Met (25 nmol) was first decarboxylated by incubating (1 or 2 h, 37°C) in 0.1 mL of 0.2 M succinate-NaOH buffer, pH 5.0, containing 1 mM pyridoxal 5'-phosphate and 11 mg of *D. erythrosora* acetone powder. The reaction mixture was then applied to 1-mL Dowex-1 (OH^-) and BioRex-70 (H^+) columns arranged in series. After washing both columns with water, [^{35}S]MTP-amine was eluted from the BioRex-70 column with 5 mL of 1 N HCl and lyophilized. It was then treated (110°C, 4 h) with 0.3 mL of 6 N HCl containing 50 μmol of MeOH to give [^{35}S]DMSP-amine, which was isolated by TLC on 0.25-mm silica-gel G plates (Machery-Nagel, Düren, Germany) developed with MeOH:acetone:concentrated HCl (90:10:4, v/v/v). The overall radiochemical yield from [^{35}S]Met was 24%; radiochemical purity was 99%, as determined by TLC and TLE. The D- and L-DMSHB (37 kBq nmol^{-1}) were prepared as described by Summers *et al.* (1998), and [*methyl*-^{14}C]DMSP-ald (0.41 kBq nmol^{-1}) was prepared as described by James *et al.* (1995b). The [^{35}S]DMSP was isolated from *S. alterniflora* leaf sections that had been given [^{35}S]Met. The [C^2H_3,C^2H_3]SMM, [$^{13}CH_3$,C^2H_3]SMM, and [C^2H_3,C^2H_3]DMSP were prepared as described by Hanson *et al.* (1994b).

Metabolism of Precursors by Leaf Tissue

Sections (about 10 x 5 mm) cut from leaves at or near full expansion were given shallow incisions spaced 1 to 2 mm apart on the whole abaxial surface. For most experiments, 0.2-g batches of sections were then incubated (cut surface down) in 6-cm Petri dishes on a 4.25-cm circle of Whatman no. 1 filter paper containing 0.5 or 1.0 mL of precursor solution. For *C. dactylon*, 0.2-g batches of shoot tips with 4 or 5 leaves were used. For the [^{35}S]SMM pulse-chase experiment, eight sections were incubated on 4 cm^2 of paper in 200 μL of [^{35}S]SMM solution and then transferred to 1 mL of water for the chase. For experiments in which DMSP-ald was analyzed, two leaf sections were incubated in 30 μL of solution. Incubation was at 25 ± 2°C, with rotary agitation at 75 rpm, in fluorescent light (PPFD, 150 μE m^{-2} s^{-1}); water was added to replace that lost by evapotranspiration. Sections were rinsed before extraction or transfer to chase media as follows: for time-course experiments, for 15 s in water; when DMSP-ald was to be analyzed, for 15 s in 10 mL of a 10^{-5} M solution of the corresponding unlabeled compound; and for other ^{35}S-labeling experiments, for 5 to 15 min in 10 mL of a 10^{-4} M solution of the corresponding unlabeled compound. The washings were pooled with the remaining incubation medium, and a sample was counted to determine ^{35}S uptake. For experiments with [$^{13}CH_3$,C^2H_3]SMM, DMSP for fast-atom-bombardment MS analysis was isolated as described by Hanson *et al.* (1994b). For all other experiments, the extraction procedures, the ion-exchange-fractionation methods, and the TLC and TLE systems used were as described by James *et al.* (1995b).

Mass Spectrometry

The DMSP was analyzed without derivatization by fast-atom-bombardment MS, employing the hexaethylene glycol/K^+ nonofluorobutylsulfonate matrix described

previously (Hanson *et al.*, 1994b). The SMM and DMSP-amine were analyzed without derivatization by MALDI-MS; the instrumentation and procedures were as described by Trossat *et al.* (1998), except that fluorosilicic acid was omitted from the matrix used to analyze DMSP-amine. These MS analyses were carried out at Michigan State University by Douglas A. Gage and Tun-Li Shen.

Computer Modeling of ^{35}S-Labeling Data

The computer model used was that described by Mayer *et al.* (1990), except that programs were written in Microsoft Visual Basic 5.0. Programs were written by David Rhodes at Purdue University and used a 0.06-min iteration interval to generate simulations of the labeling patterns of metabolites, partition pools into metabolically active and inactive (storage) components, and vary the fluxes between pools until the match between observed and simulated data was satisfactory.

Results and Discussion

Metabolism of [^{35}S]Met, [^{35}S]SMM, and [^{35}S]DMSHB

A diagnostic distinction between the algal- and Compositae-type DMSP synthesis pathways is that if radiotracer Met is supplied, the major labeled intermediate is DMSHB in the former and SMM in the latter (Hanson *et al.*, 1994b; Gage *et al.*, 1997). Therefore, [^{35}S]Met was fed to *S. alterniflora* leaf tissue and the labeling of DMSHB, SMM, and DMSP was monitored (Figure 11A). As in Compositae, no ^{35}S was detected in DMSHB but SMM acquired label rapidly and lost it as the [^{35}S]Met was metabolized, as expected for an intermediate. However, unlike the pattern in Compositae, DMSP-amine acquired and lost ^{35}S in a way that suggested that it too could be an intermediate (Figure 11B).

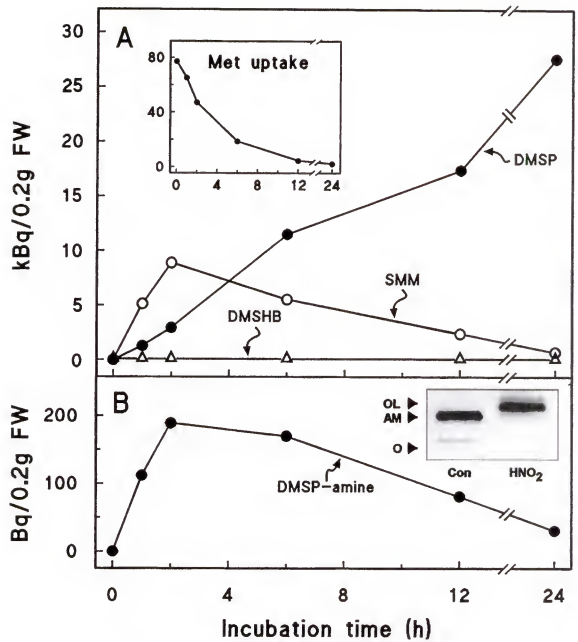

Figure 11. Labeling patterns of metabolites in *S. alterniflora* leaf sections supplied with [^{35}S]Met (77 Bq [10 nmol] per 0.2 g fresh weight).
A. SMM, DMSHB, and DMSP. Labeling of DMSHB was below the detection limit (0.03 kBq) at all times. Inset shows [^{35}S]Met uptake from the medium. B. DMSP -amine. [^{35}S]DMSP-amine was identified by co-migration with authentic DMSP -amine in TLC (systems 1 and 2 of James *et al.* [1995b]) and TLE (system 1 of James *et al.* [1995b]), and by deamination to 3-dimethylsulfoniopropanol with nitrous acid (Zappia *et al.*, 1969) as shown in the inset. The inset is an autoradiograph of a TLC -system 1 separation of [^{35}S]DMSP-amine from *S. alterniflora*, before (Con) and after (HNO_2) nitrous acid treatment; the positions of the origin (O), DMSP-amine (AM), and 3-dimethylsulfoniopropanol (OL) are marked. FW, Fresh weight.

Indeed, the decarboxylation product of SMM is DMSP-amine [$(CH_3)_2S^+CH_2CH_2CH_2$-NH_3^+]. The [^{35}S]DMSP-amine pool was far smaller than the [^{35}S]SMM pool, its peak ^{35}S content (between 1 and 6 h) being about 2.5% that of SMM (Figure 11); two similar experiments gave peak values of 2.9% and 5.0% (not shown).

Another diagnostic difference between the two known DMSP synthesis pathways is the extent of conversion of supplied SMM or DMSHB to DMSP. In this respect, *S. alterniflora* again resembled Compositae, since leaf segments readily converted [^{35}S]SMM to DMSP, but did not metabolize either the D- or L-enantiomer of DMSHB (Table VI).

Table VI. Conversion of supplied SMM but not DMSHB to DMSP by *S. alterniflora* leaf sections.
Leaf sections (0.2 g fresh weight) were incubated for 24 h with a tracer dose (0.2 to 1.0 nmol) of [^{35}S]SMM or [^{35}S]DMSHB. The amount of ^{35}S was 19.2 to 19.6 kBq in all cases. Uptake was calculated from the disappearance of ^{35}S from the medium. The experiment was repeated using 100-nmol doses of ^{35}S precursors, with very similar results. The values shown are per 0.2 g fresh weight and have been corrected for recovery.

^{35}S Precursor	^{35}S Uptake	[^{35}S]DMSHB	[^{35}S]DMSP
		kBq	
L-SMM	18.9	<0.03	6.9
L-DMSHB	11.8	11.2	<0.1
D-DMSHB	6.1	5.7	<0.1

Evidence from Stable Isotope Labeling that SMM and DMSP-amine are Intermediates

The ^{35}S-labeling data discussed above are consistent with the following reaction sequence: Met → SMM → → DMSP. However, because Met and SMM are potentially interconvertible via the SMM cycle (Mudd and Datko, 1990), they are also consistent with the sequence: SMM ↔ Met → → DMSP. To distinguish between these alternatives, leaf segments were given SMM labeled with ^{13}C in one methyl group and ^{2}H in the other, and the DMSP formed was analyzed (Raven, 1987). Only [$^{13}CH_3$,C^2H_3]DMSP was detected (Table VII). This shows that SMM was converted to DMSP directly, not via Met, because conversion via Met would give $^{13}CH_3$, $^{13}CH_3$-, $^{13}CH_3$,C^2H_3- and C^2H_3,C^2H_3-labeled species of DMSP in a 1:2:1 ratio. These results therefore confirm that SMM is an intermediate of DMSP synthesis in *S. alterniflora*. They also indicate that flux through the SMM cycle in this plant is small compared to the flux to DMSP.

To supplement the radiolabeling evidence that SMM is converted to DMSP via DMSP-amine, leaf segments were fed unlabeled or C^2H_3,C^2H_3-labeled SMM, and the DMSP-amine sought by a sensitive MALDI-MS method. Strong signals appeared at m/z 120 or 126, corresponding to unlabeled and C^2H_3,C^2H_3-labeled DMSP-amine, respectively (Figure 12). Confirmation that these peaks represent DMSP-amine was obtained through MALDI-post-source decay experiments; these showed the expected fragment at m/z 58, formed from the precursor ion by neutral loss of $(CH_3)_2S$ or $(C^2H_3)_2S$. That [C^2H_3,C^2H_3]SMM gave rise to little or no [C^2H_3]DMSP-amine (m/z 123) (Figure 12B) indicates that the dimethylsulfonium group enters DMSP-amine as a unit, which is consistent with direct conversion of SMM to DMSP-amine by decarboxylation.

The MALDI-MS technique was also used to quantify the endogenous SMM pool in *S. alterniflora*, with an internal standard of $[C^2H_3,C^2H_3]$SMM. Duplicate analyses gave values of 143 and 160 nmol g^{-1} fresh weight. Additionally, a signal attributable to endogenous DMSP-amine was detectable by MALDI-MS; it was too small to quantify accurately, but indicated that the DMSP-amine level was not more than 20 nmol g^{-1} fresh weight, *i.e.* far lower than the SMM level.

Table VII. Labeling of DMSP synthesized by *S. alterniflora* leaf sections given $[^{13}CH_3,C^2H_3]$SMM.
Leaf sections (0.2 g fresh weight) were incubated with 5.0 μmol of $[^{13}CH_3,C^2H_3]$SMM (experimental samples) or unlabeled SMM (controls) for 24 or 48 h. The intensities of the labeled DMSP ions are expressed relative to that of endogenous unlabeled DMSP (*m/z* 135, 100%); the endogenous DMSP level was 23 ± 1 μmol g^{-1} fresh weight (mean ± SE, $n = 4$). All signals were corrected for background noise from the matrix, and those at *m/z* 141 were further corrected for the contribution from the natural-abundance isotope peaks associated with $[^{13}CH_3,C^2H_3]$DMSP (*m/z* 139). Because labeled DMSP yields at 24 and 48 h did not differ significantly, results for both times were pooled. Data are means from eight experimental samples and four controls, and were subjected to analysis of variance. That all of the values for control sections given unlabeled SMM are close to 0 confirms the validity of the corrections applied. MS analyses were carried out at Michigan State University by Douglas A. Gage and Tun-Li Shen.

Precursor	Relative Intensity of DMSP Ions		
	$^{13}CH_3,^{13}CH_3$ (*m/z* 137)	$^{13}CH_3,C^2H_3$ (*m/z* 139)	C^2H_3,C^2H_3 (*m/z* 141)
		%	
$[^{13}CH_3,C^2H_3]$SMM	-0.27	7.63	0.10
Unlabeled SMM	-0.01	0.09	0.02
$LSD_{0.05}$	0.45	1.80	0.64

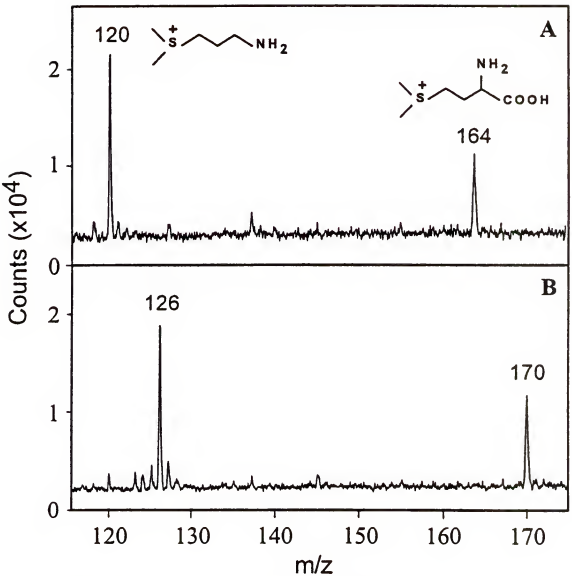

Figure 12. MALDI-MS analysis of the base fractions from *S. alterniflora* leaf segments (0.2 g fresh weight) incubated for 6 h with 5.0 μmol of unlabeled SMM (A) or $[C^2H_3,C^2H_3]$SMM (B).
The peaks at *m/z* 164 and 170 correspond to the unlabeled SMM and $[C^2H_3,C^2H_3]$-SMM supplied, respectively, and those at *m/z* 120 and 126 correspond to unlabeled and C^2H_3,C^2H_3-labeled DMSP-amine formed during the experiment, respectively. In these spectra, matrix background peaks are largely suppressed, but residual signals can be seen at *m/z* 123, 137, and 146. The relative response of DMSP-amine and SMM in MALDI-MS analyses under these conditions was approximately 30:1, determined by spiking the samples with known amounts of DMSP-amine and SMM (data not shown). In leaf segments not given SMM, the intensities of the peaks corresponding to DMSP-amine and SMM were respectively about 5 and 15% of those in A. No peaks attributable to DMSP-amine were detected in the SMM substrates supplied to the leaf segments.

Labeling Kinetics of DMSP-amine During and After a Pulse of [^{35}S]SMM

A pulse-chase experiment with [^{35}S]SMM was used to further test the possibility that DMSP-amine is a DMSP synthesis intermediate (Figure 13). In the first minutes of the pulse, ^{35}S accumulated in DMSP-amine more rapidly than in DMSP (Figure 13B, inset), which fits qualitatively with DMSP-amine being an intermediate. During the chase, DMSP-amine lost label as the [^{35}S]SMM was depleted and [^{35}S]DMSP synthesis slowed, again consistent with its being an intermediate. The data also confirm that there is only a minor SMM → Met flux via the SMM cycle, because little ^{35}S accumulated in free Met at any time (< 0.1 kBq; not shown) and labeling of the insoluble fraction, a maximum estimate of protein-bound [^{35}S]Met, was small relative to that of DMSP (Figure 13A).

Metabolism of [^{35}S]SMM in Grasses that Do Not Accumulate DMSP

If DMSP-amine is an intermediate specific to the DMSP synthesis pathway, then species that lack DMSP should not synthesize it from supplied SMM. This prediction was tested by comparing the metabolism of [^{35}S]SMM in *S. alterniflora* with that in eight grasses that were shown to lack detectable amounts of DMSP (Table VIII). These grasses lacking DMSP were chosen to include another species of *Spartina* (*S. patens*), and otherwise for maximum diversity; together, they represented all five subfamilies of Gramineae. The DMSP-free grasses took up 56 to 97% of the [^{35}S]SMM supplied, but none produced a detectable quantity of [^{35}S]DMSP-amine or more than a trace of [^{35}S]DMSP (Table VIII). This incapacity to form DMSP-amine among a wide range of Gramineae that do not accumulate DMSP indicates that DMSP-amine is an intermediate in the DMSP pathway, and not simply a common minor metabolite of grasses.

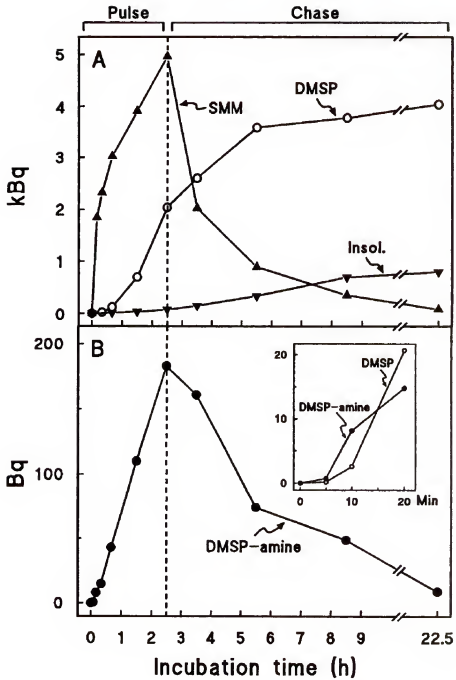

Figure 13. Labeling kinetics of SMM, DMSP, and insoluble material (Insol., A), and DMSP-amine (B) in a pulse-chase experiment with [^{35}S]SMM. Batches of eight *S. alterniflora* leaf sections (approximately 0.1 g fresh weight) were supplied with 18.5 kBq (0.16 nmol) of [^{35}S]SMM; after 2.5 h they were rinsed for 15 s and transferred to water. That more ^{35}S was lost from SMM during the chase than appeared in other products may be ascribed to efflux of [^{35}S]SMM present in the apoplast. The inset in B is an expanded -scale plot of the labeling of DMSP-amine and DMSP at early times; note that DMSP-amine is initially more heavily labeled than DMSP.

Table VIII. [^{35}S]DMSP-amine is not a metabolite of [^{35}S]SMM in grasses that lack DMSP.
Leaf tissue (0.2 g fresh weight) of eight grasses shown not to accumulate DMSP was incubated for 6 h with 38.9 kBq (0.12 nmol) of [^{35}S]SMM; *S. alterniflora* was included as a benchmark. Uptake was calculated from ^{35}S disappearance from the medium. The radioactivity values shown are per 0.2 g fresh weight and have been corrected for recovery. Endogenous DMSP contents were determined on duplicate 30-mg fresh weight leaf samples. The comparison between *S. alterniflora* and *S. patens* was repeated, with similar results. FW, fresh weight.

Species	Endogenous DMSP	^{35}S Uptake	[^{35}S]DMSP-amine	[^{35}S]DMSP
	μmol g^{-1} FW	*kBq*	*Bq*	*Bq*
Spartina alterniflora	29.0	18.3	389	8180
Spartina patens	<0.03	28.6	<30	<30
Triticum aestivum	<0.03	37.0	<30	<30
Zea mays	<0.03	36.6	<30	90
Bambusa glaucescens	<0.03	36.3	<30	<30
Oplismenus compositus	<0.03	37.6	<30	<30
Cynodon dactylon	<0.03	35.1	<30	<30
Pennisetum purpureum	<0.03	36.8	<30	70
Cortaderia selloana	<0.03	21.6	<30	<30

Metabolism of [^{35}S]DMSP-amine in *S. alterniflora* and Grasses Lacking DMSP

As an intermediate of DMSP synthesis, supplied DMSP-amine should be readily converted to DMSP by *S. alterniflora*. Figure 14 confirms that this was true for both

tracer and substrate doses of [^{35}S]DMSP-amine. It also shows that the eight grasses lacking DMSP produced relatively little [^{35}S]DMSP, although their [^{35}S]DMSP-amine uptake was generally greater than that of *S. alterniflora*. This supports the view that DMSP-amine is a characteristic intermediate of the DMSP pathway in *S. alterniflora*, and underscores the distinction between this species and Compositae, in which DMSP-amine is not as readily metabolized to DMSP (James *et al.*, 1995b). That the DMSP-free grasses made a little [^{35}S]DMSP agrees with previous data for *T. aestivum* and for dicot species that lack DMSP (James *et al.*, 1995b). This seemingly nonspecific DMSP production may be caused by the tandem action of diamine oxidase and ω-aminoaldehyde dehydrogenase, both of which occur in grasses and dicots. Both enzymes can attack substrates with a dimethylsulfonium group (Bardsley *et al.*, 1971; Awal *et al.*, 1995; Suzuki, 1996; Trossat *et al.*, 1997).

A remarkable feature of the metabolism of [^{35}S]DMSP-amine in *S. alterniflora* was that whether the amount taken up was 0.05 or 1,000 nmol, about 20% of it was metabolized to [^{35}S]DMSP (Figure 14). Such a high capacity for metabolizing DMSP -amine to DMSP suggests that unlabeled DMSP-amine might not act as an effective trap for label coming from [^{35}S]SMM. This proved to be the case; combining a 3×10^4-fold excess of unlabeled DMSP-amine with tracer [^{35}S]SMM resulted in a high internal DMSP-amine level, but did not cause ^{35}S to accumulate in DMSP-amine (Table IX). The unlabeled DMSP-amine reduced [^{35}S]SMM uptake (and [^{35}S]DMSP synthesis), but this is unlikely to have masked a trapping effect because the size of the internal [^{35}S]SMM pool hardly changed (Table IX).

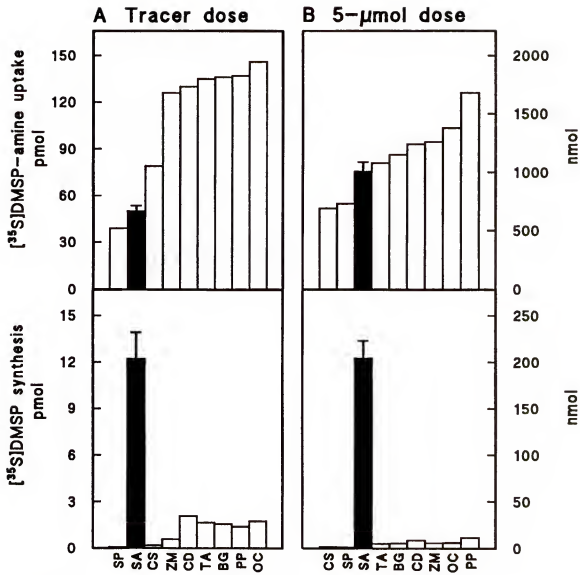

Figure 14. Uptake and conversion to [^{35}S]DMSP of tracer (A) or substrate-level (B) doses of [^{35}S]DMSP-amine.
Leaf tissue (0.2 g fresh weight) of nine grass species was incubated with 140 ± 5 pmol (tracer) or 5.0 µmol (substrate-level) of [^{35}S]DMSP-amine for 6 h. Uptake was calculated from disappearance of ^{35}S from the medium. Species are arranged in order of ascending label uptake. Species are as follows: SP, *Spartina patens*; SA, *Spartina alterniflora*; CS, *Cortaderia selloana*; ZM, *Zea mays*; CD, *Cynodon dactylon*; TA, *Triticum aestivum*; BG, *Bambusa glaucescens*; PP, *Pennisetum purpureum*; and OC, *Oplismenus compositus*. All species except *S. alterniflora* lacked detectable DMSP (Table VIII). The data for *S. alterniflora* are means and SE for four similar experiments; those for other species are single determinations.

Table IX. DMSP-amine does not act as a trapping pool for label from [^{35}S]SMM. *Spartina alterniflora* leaf sections (0.2 g fresh weight) were incubated for 4 h with 18.5 kBq (0.15 nmol) of [^{35}S]SMM, with or without a 5-µmol trapping pool of unlabeled DMSP-amine. The size of the DMSP-amine pool in the tissue was estimated in a parallel experiment in which leaf sections were incubated with 5 µmol (18.5 kBq) of [^{35}S]DMSP-amine for 4 h. The values shown are per 0.2 g fresh weight and have been corrected for recovery. The experiment was repeated using an 8-h incubation time, with similar results.

DMSP-amine Trap		[^{35}S]SMM	Label Distribution		
Supplied	In Tissue	Uptake	SMM	DMSP-amine	DMSP
nmol		*kBq*	*kBq*	*Bq*	*kBq*
0	0	5.22	1.96	111	2.52
5000	568	2.44	1.48	126	0.78

Evidence For DMSP-ald as an Intermediate

On comparative biochemical grounds, the most plausible route between DMSP -amine and DMSP would be via DMSP-ald. Therefore [^{35}S]DMSP-ald was investigated as a metabolite of [^{35}S]SMM or [^{35}S]DMSP-amine. During a pulse of either precursor, DMSP-ald acquired detectable ^{35}S, and lost much of it during a subsequent chase period (Table X). These data are consistent with DMSP-ald being an intermediate in DMSP synthesis. The data for the [^{35}S]SMM pulse indicate that the DMSP-ald pool is very small (about 2.5% the size of the DMSP-amine pool) and turns over very fast (at least once every 100 s).

Table X. Labeling of DMSP-ald from [^{35}S]SMM or [^{35}S]DMSP-amine in *S. alterniflora*. Pairs of leaf segments were supplied with a pulse of [^{35}S]SMM (77 kBq, 0.99 nmol) or [^{35}S]DMSP-amine (35 kBq, 1.33 nmol) for 3 h, rinsed for 15 s, and then transferred to water for a 9-h chase period. DMSP-ald was analyzed as the corresponding alcohol after reduction with $NaBH_4$ (Finley and Wilson Jr., 1970; also see "Materials and Methods"). A correction was made for traces of alcohol detectable in controls that were not treated with $NaBH_4$. That more ^{35}S was lost from SMM or DMSP-amine during the chase than appeared in DMSP may be ascribed primarily to efflux of unabsorbed label from the apoplast. The values shown are per pair of segments and have been corrected for recovery. The experiment was repeated, with similar results.

^{35}S Precursor	Treatment	Label Distribution			
		SMM	DMSP-amine	DMSP-ald	DMSP
		kBq	*Bq*	*Bq*	*kBq*
SMM	Pulse	69.3	936	24.1	2.44
	Pulse-Chase	23.8	466	8.1	13.0
DMSP-amine	Pulse	--[a]	23400	11.8	1.41
	Pulse-Chase	--	3990	1.1	2.74

[a]--, Below detection limit (0.05 kBq).

Modeling of DMSP Synthesis in *S. alterniflora*

The kinetic data of Figures 11 and 13, and results from an additional pulse-chase experiment with [^{35}S]Met (not shown), were used to develop the model summarized in Figure 15. For each experiment, flux rates and pool sizes were progressively adjusted until a close match between observed and simulated values was obtained (overall r^2 = 0.991, n = 80). Means and SE values of these best-fit estimates for the three independent

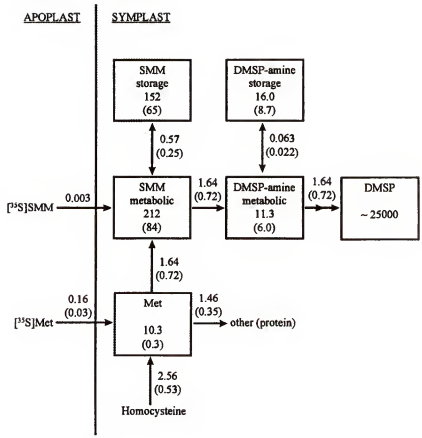

Figure 15. A model of DMSP synthesis from Met in *S. alterniflora*, based on computer -assisted analyses of the data shown in Figures 11 and 13 plus an additional pulse-chase experiment with [^{35}S]Met (not shown).
Values in boxes are mean pool sizes (nmol g^{-1} fresh weight) and those next to arrows are mean flux rates (nmol min^{-1} g^{-1} fresh weight). Values in parentheses are SE. The DMSP pool size shown is based on the experimental values given in Tables VII and VIII because modeling did not permit estimation off this parameter (modeling permitted estimation only of the amount of label that accumulated in DMSP, which is independent of the pool size assumed). The rates of [^{35}S]SMM and [^{35}S]Met uptake from the apoplast (assumed to equilibrate rapidly with the medium) apply only to the pulse phase of pulse-chase experiments. During the chase the uptake rates were reduced by 20- to 40-fold, depending on the dilution of the medium plus apoplastic precursor pools achieved during the chase. For the experiment shown in Figure 11, which did not involve a chase, the [^{35}S]Met uptake rate was assumed to be 0.192 nmol min^{-1} g^{-1} fresh weight initially, and thereafter to decline in direct proportion to the size of the exogenous [^{35}S]Met pool (see Figure 11A, inset). For all experiments it was necessary to postulate that there was a large endogenous flux of (unlabeled) homocysteine to Met, SMM, DMSP-amine, and DMSP, and that portions of the SMM and DMSP-amine pools were sequestered in "storage" compartments. These storage pools were postulated to be in slow equilibrium with the "metabolically active" pools that participated as intermediates in DMSP synthesis. Labeling of DMSP-ald (an intermediate between DMSP-amine and DMSP) was not considered. Computer programs were written by David Rhodes at Purdue University, who also modeled the data.

experiments are shown in Figure 15. For simplicity, the model did not include a DMSP -ald intermediate because the pool size of DMSP-ald was so small as to have no effect on estimates of the DMSP-amine → DMSP flux.

These modeling results show that the observed labeling patterns are quantitatively consistent with a pathway of DMSP synthesis in which DMSP-amine is an intermediate. A noteworthy point is that the model postulates "storage" pools of both SMM and DMSP-amine in addition to "metabolically active" pools, the flux rates between these pools being low compared with the rate of DMSP synthesis. One possible physical basis for this is that SMM conversion to DMSP occurs in the chloroplast, as in *W. biflora* (Trossat *et al.*, 1996); in this case, the metabolic pools of SMM and DMSP-amine would be chloroplastic and the storage pools extrachloroplastic (cytosolic and/or vacuolar). For DMSP-amine, which is doubly and positively charged at cellular pH, another possible basis for the storage component is reversible adsorption to negative charges on macromolecules, as occurs with di- and polyamines (Kumar *et al.*, 1997, and refs. cited therein).

The mean total pool size of SMM estimated from the model is more than twice that measured by MALDI-MS. However, the modeling results indicated appreciable variation in pool sizes and fluxes between different batches of *S. alterniflora* leaves (see the SE values in Figure 15), and the range of model-derived total SMM pool sizes (90 to 511 nmol g^{-1} fresh weight) encompasses the experimental value of approximately 150 nmol g^{-1} fresh weight. The model-estimated total DMSP-amine pool has a mean value of about 7% of the SMM pool, which is broadly consistent with the MALDI-MS estimates (see "Evidence from Stable Isotope Labeling that SMM and DMSP-amine are

Intermediates"). When expressed per day, the model-estimated flux rate to DMSP is 2.36 μmol d^{-1} g^{-1} fresh weight, which is equivalent to about 10% of the total DMSP pool.

The model was used to estimate the SMM and DMSP-amine pool sizes, plus the SMM → DMSP-amine and DMSP-amine → DMSP fluxes, in two additional labeling experiments. The first experiment was that described in Table IX, with and without a trapping pool of DMSP-amine. The results with no trapping pool were satisfactorily accommodated by using flux and pool size values that fell within the ranges given in Figure 15. When DMSP-amine was supplied, the model gave a good fit to experimental data if (a) the exogenous DMSP-amine was postulated to enter the DMSP synthesis pathway by passing via the storage pool, (b) the DMSP-amine storage pool expanded massively from approximately 5 to 2,700 nmol g^{-1} fresh weight, (c) the DMSP-amine metabolic pool expanded from 4 to 75 nmol g^{-1} fresh weight, and (d) the DMSP-amine → DMSP flux increased from 1 to 3.78 nmol min^{-1} g^{-1} fresh weight. These assumptions seem biologically reasonable; note that assumption (a) is consistent with exogenously supplied DMSP-amine passing through a cytosolic (storage) pool en route to a chloroplastic (metabolic) pool. The model thus provides an acceptable quantitative explanation for the failure of added DMSP-amine to trap label from SMM.

The second additional experiment to which the model was applied (not shown) involved supplying a large dose of [^{35}S]SMM (5 μmol/200 mg fresh weight) for 4 h. The data were best accommodated by postulating an expansion of the metabolic pool of SMM from 75 to 2,700 nmol g^{-1} fresh weight coupled with an increase in the SMM → DMSP -amine flux from 1 to 4.8 nmol min^{-1} g^{-1} fresh weight. This resulted in an expansion of

the metabolic DMSP-amine pool from 4 to 100 nmol g^{-1} fresh weight and an increase in the DMSP-amine → DMSP flux from 1 to 4.4 nmol min^{-1} g^{-1} fresh weight.

Taken together with the data summarized in Figure 15, these modeling results revealed hyperbolic relationships between the SMM metabolic pool size and the SMM → DMSP-amine flux, and between the DMSP-amine metabolic pool size and the DMSP -amine → DMSP flux (Figure 16). Such saturable kinetics are to be expected given that the fluxes are enzyme-mediated, and the fact that they are observed supports the validity of the model. Double-reciprocal plots gave apparent K_m values for SMM and DMSP -amine of 310 and 5.8 nmol g^{-1} fresh weight, respectively. These values cannot be converted to concentrations because the size of the compartment(s) containing the metabolic pools of SMM and DMSP-amine are unknown.

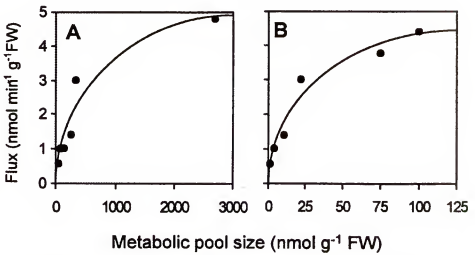

Figure 16. Model-derived relationships between metabolic pool sizes and fluxes for SMM and the SMM → DMSP-amine flux (A), and for DMSP-amine and the DMSP-amine → DMSP flux (B).
Each data point corresponds to metabolic pool size and flux values obtained using the model shown in Figure 15. The experimental data input for the model came from Figures 11 and 13, from Table IX, and from the two additional experiments described in the text. FW, fresh weight. Computer programs were written by David Rhodes at Purdue University, who also modeled the data.

Conclusions

In this study, the DMSP synthesis pathway in *S. alterniflora* has been shown to be unlike that in marine algae, and to resemble that in Compositae by having SMM and DMSP-ald as intermediates. However, it does differ crucially from that of Compositae in that SMM is converted to DMSP-ald via a pool of free DMSP-amine (Figure 17). The presence of a free DMSP-amine intermediate is supported by the labeling kinetics of

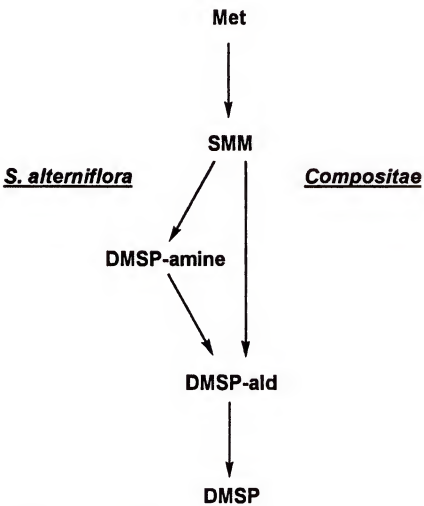

Figure 17. DMSP synthesis from Met in *S. alterniflora* and species of Compositae. Many flowering plants can carry out the *S*-methylation of Met to SMM and the oxidation of DMSP-ald to DMSP. Only the conversion of SMM to DMSP-ald is unique to DMSP synthesis.

DMSP-amine when [^{35}S]Met or [^{35}S]SMM is fed, also by the formation of [C^2H_3,C^2H_3]-DMSP-amine from [C^2H_3,C^2H_3]SMM, and by the ready conversion of supplied DMSP -amine to DMSP. That DMSP-amine is taken up but does not trap label coming from SMM can be explained by its relatively poor access to the metabolic pool and by the high capacity for converting DMSP-amine to DMSP.

Given the prevalence among angiosperms of both SMM and enzymes able to oxidize DMSP-ald (Giovanelli *et al.*, 1980; Trossat *et al.*, 1997), it is the ability to convert SMM to DMSP-ald that distinguishes plants that produce DMSP from those that do not (Figure 17). Because *S. alterniflora* and Compositae evidently differ in how they carry out this key conversion, it seems likely that their DMSP pathways had independent evolutionary origins. Dimethylsulfoniopropionate synthesis may thus have evolved at least three times, once each in algae, dicots, and monocots. With respect to evolution of the pathway in *S. alterniflora*, two novel enzymes would presumably be required: an SMM decarboxylase and a DMSP-amine oxidase, dehydrogenase, or aminotransferase. In this regard, it is worth noting that SMM is an analog of *S*-adenosyl-L-Met (AdoMet), and that AdoMet decarboxylases are ubiquitous (Kumar *et al.*, 1997). Likewise, DMSP -amine is an analog of a diamine, and diamine oxidases occur widely in plants (Suzuki, 1996). It is therefore easy to imagine how novel enzymes able to decarboxylate SMM and able to oxidize DMSP-amine might have originated.

CHAPTER 5
BIOCHEMICAL EVIDENCE FOR TWO NOVEL ENZYMES IN THE BIOSYNTHESIS OF 3-DIMETHYLSULFONIOPROPIONATE IN *SPARTINA ALTERNIFLORA*

Chapter Summary

The highly soluble zwitterion, 3-dimethylsulfoniopropionate (DMSP), is an effective osmoprotectant accumulated by the cordgrass *Spartina alterniflora* and other salt-tolerant plants. Previous *in vivo* isotope tracer and metabolic modeling studies demonstrated that *S. alterniflora* synthesizes DMSP via the route *S*-methyl-Met (SMM) → 3-dimethylsulfoniopropylamine (DMSP-amine) → 3-dimethylsulfoniopropion-aldehyde (DMSP-ald) → DMSP, and indicated that the first reaction requires a far higher substrate concentration than the second to attain half-maximal rate. As neither of these reactions is known from other organisms, two novel enzymes are predicted. Two corresponding activities were identified in *S. alterniflora* leaf extracts using specific radioassays. The first, SMM decarboxylase (SDC), strongly prefers the L-enantiomer of SMM, is pyridoxal 5'-phosphate (PLP)-dependent, generates equimolar amounts of CO_2 and DMSP-amine, and has a high apparent K_m (≈ 18 mM) for its substrate. The second enzyme, DMSP-amine oxidase (DOX), requires O_2 for activity, shows an apparent K_m for DMSP-amine of 1.8 mM, and is not accompanied by DMSP-amine dehydrogenase or transaminase activity. Very little SDC or DOX activity was found in grasses lacking

DMSP. These data indicate that SDC and DOX are the predicted novel enzymes of DMSP synthesis.

Introduction

The tertiary sulfonium compound 3-dimethylsulfoniopropionate (DMSP) is accumulated by certain salt-tolerant angiosperms and many marine algae (Malin and Kirst, 1997; McNeil *et al.*, 1999). Dimethylsulfoniopropionate is structurally analogous to a betaine and, like betaines, functions as a cytoplasmic compatible solute or osmoprotectant and contributes to adaptation to osmotic and freezing stresses (Rhodes and Hanson, 1993; Karsten *et al.*, 1996; Vianney *et al.*, 1998). Dimethylsulfoniopropionate differs from betaines in containing sulfur instead of nitrogen and, in DMSP-accumulating plants, appears to act as a substitute for betaines when nitrogen is scarce (Colmer *et al.*, 1996; Cooper and Hanson, 1998). Engineering for the accumulation of betaines or other osmoprotectants can improve osmotic or freezing stress resistance (Holmberg and Bülow, 1998; Nuccio *et al.*, 1999). Since nitrogen often limits crop growth, and DMSP accumulation does not require nitrogen, DMSP synthesis is an attractive target for the engineering of stress resistance in low-nitrogen environments (McNeil *et al.*, 1999).

The biosynthesis of DMSP is also environmentally important because DMSP is the main biogenic precursor of dimethylsulfide (DMS) released to the atmosphere from the oceans (Malin and Kirst, 1997) and is a likely precursor of DMS coming from land (Dacey *et al.*, 1987; Paquet *et al.*, 1994). Biogenic DMS plays a pivotal role in the global sulfur cycle, affects the pH of precipitation, and is believed to contribute to the regulation of global climate (Malin, 1996).

In vivo isotope labeling and modeling studies (Kocsis *et al.*, 1998) demonstrated that DMSP biosynthesis in the saltmarsh cordgrass *Spartina alterniflora* proceeds from L-Met via *S*-methyl-Met (SMM), 3-dimethylsulfoniopropylamine (DMSP-amine), and 3-dimethylsulfoniopropionaldehyde (DMSP-ald) (Figure 18). The first and last steps in this pathway are the same as in the dicot *Wollastonia biflora*, but the central part is not. In *W. biflora*, SMM is converted directly to DMSP-ald without the formation of DMSP -amine, most likely via a transamination/decarboxylation mechanism (Hanson *et al.*, 1994b; James *et al.*, 1995b; Rhodes *et al.*, 1997). All angiosperms appear to produce SMM (Mudd and Datko, 1990; Bourgis *et al.*, 1999), and to have enzymes that can catalyze the conversion of DMSP-ald to DMSP (Trossat *et al.*, 1997; Vojtěchová *et al.*, 1997). It is thus the conversion of SMM to DMSP-ald that is unique to DMSP synthesis, and *S. alterniflora* appears to have evolved specific enzymes that mediate this conversion. Besides predicting the existence of novel enzymes in *S. alterniflora* that convert SMM to DMSP-amine, and DMSP-amine to DMSP-ald, the *in vivo* tracer and modeling studies predicted that, of these enzymes, the first has a much higher K_m for its sulfonium substrate than the second (Kocsis *et al.*, 1998).

Reported here are the identification and initial characterization of, as well as the assay procedures for, two enzymes from *S. alterniflora* that catalyze the SMM → DMSP -amine and DMSP-amine → DMSP-ald steps. The first is SMM decarboxylase (SDC). The second is DMSP-amine oxidase (DOX). Comparative biochemical data indicate that both are specific to the DMSP pathway.

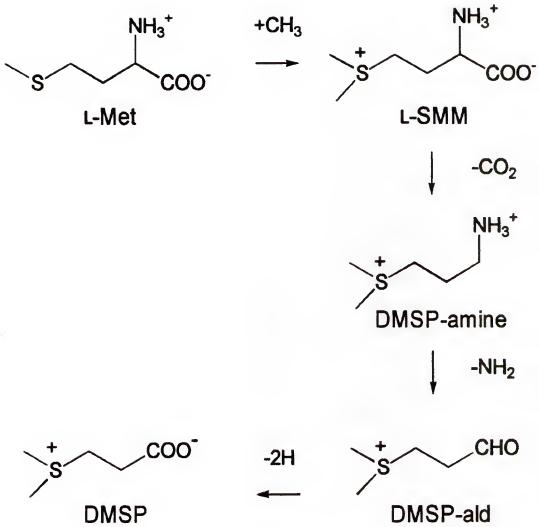

Figure 18. The DMSP biosynthesis pathway in *S. alterniflora*. Only the decarboxylation of SMM and conversion of DMSP-amine to DMSP-ald are unique to DMSP biosynthesis. Enzymes able to catalyze the other two steps are widespread in angiosperms.

Materials and Methods

Plants

The *S. alterniflora* Loisel. and *S. patens* (Ait.) Muhl. were collected from Crescent Beach, Florida and grown in undrained pots in a naturally-lit greenhouse at 15 to 35°C. The *S. alterniflora* was grown in a 10:1 (w/w) mixture of sand and potting soil (Fafard mix 3-B, Conrad Fafard, Agawam, MA) according to Otte and Morris (1994). The *S. patens* (Nellis, 1994) was grown in soil from the collection site. Maize (*Zea mays* L. cv NK 508) was grown in potting soil in the same greenhouse conditions. Wheat (*Triticum aestivum* L. cvs Florida 310 or Bob White) was grown in potting soil in a chamber with a 12-h photoperiod (200-300 $\mu E\ m^{-2}\ s^{-1}$; 25°C day/22°C night). Plants were watered as required and fertilized weekly with Peters soluble fertilizer (20-20-20, NPK; Scotts-Sierra Horticultural Products, Marysville, OH).

Chemicals and Radiochemicals

The L-[^{35}S]Met (43.5 MBq $nmol^{-1}$), L-[U-^{14}C]Met (9.56 kBq $nmol^{-1}$), and [^{14}C]formate (1.79 kBq $nmol^{-1}$) were obtained from NEN Life Science Products (Boston, MA), D-[1-^{14}C]Met (2.07 kBq $nmol^{-1}$) was from Moravek Biochemicals (Brea, CA), and L-[1-^{14}C]Met (2.03 kBq $nmol^{-1}$) was from American Radiolabeled Chemicals (St. Louis, MO). Specific radioactivities were adjusted with unlabeled compounds. The 3-methylthiopropylamine (MTP-amine) from Chem Service (West Chester, PA) was used to prepare DMSP-amine as described by Kocsis *et al.* (1998). The DMSP was from Research Plus (Bayonne, NJ). The iodide form of L-SMM (Sigma, St. Louis, MO) was converted to the hydrochloride form by ion exchange (Kocsis *et al.*, 1998). Ion-exchange resins were purchased from Bio-Rad Laboratories (Hercules, CA).

Radiochemical Syntheses

The D- and L-[1-^{14}C]SMM (2.07 and 2.03 kBq nmol^{-1}, respectively) were synthesized from D- and L-[1-^{14}C]Met using methanol as the methylating agent (Gage *et al.*, 1997). The L-[^{35}S]SMM (370 kBq nmol^{-1}) was made from L-[^{35}S]Met in the same way. The L-[U-^{14}C]SMM (9.56 kBq nmol^{-1}) was synthesized from L-[U-^{14}C]Met and *S*-adenosyl-L-Met (AdoMet) using Met *S*-methyltransferase (MMT) (Trossat *et al.*, 1996). After the syntheses, labeled SMM was isolated (> 98% radiochemical purity) using thin-layer electrophoresis (TLE) system 2 (James *et al.*, 1995b). The [^{35}S]DMSP (52-277 Bq nmol^{-1}) was isolated from *S. alterniflora* leaf sections fed with L-[^{35}S]Met or L-[^{35}S]SMM (Kocsis *et al.*, 1998). The [^{35}S]dimethylsulfoxide (DMSO) was obtained by treating [^{35}S]DMSP with 17% (w/v) NaOH for 2 h to liberate [^{35}S]DMS (White, 1982; Reed, 1983), which was trapped and converted to [^{35}S]DMSO on a 1-cm Whatman (Clifton, NJ) No. 3 paper disk containing 20 µL 30% (w/w) H_2O_2 (Kiene and Linn, 2000). The [^{35}S]DMSO was then eluted with water. To prepare [^{35}S]DMSP-amine (370-740 kBq nmol^{-1}), L-[^{35}S]Met (25 nmol) was decarboxylated by incubating (2h, 37°C, under N_2) in 0.1 mL of 0.2 M succinate-NaOH buffer, pH 5.0, containing 1 mM pyridoxal 5'-phosphate (PLP) and 11 mg autumn fern acetone powder (Kocsis *et al.*, 1998). The reaction mixture was applied to 1-mL AG-1 (OH^-) and BioRex-70 (H^+) columns arranged in series. After washing the columns with water, [^{35}S]MTP-amine and its sulfoxide were eluted from the BioRex-70 column with 5 mL of 1 N HCl, and lyophilized. The dry sample was then dissolved in 0.1 mL water plus 4 µL of 70% (w/w) thioglycolic acid and heated at 95°C for 3 h to reduce the sulfoxide to [^{35}S]MTP-amine. Excess thioglycolate

was removed by lyophilization. Methylation of [^{35}S]MTP-amine gave [^{35}S]DMSP-amine, which was isolated by thin-layer chromatography (TLC) (Kocsis *et al.*, 1998). The radiochemical yield of [^{35}S]DMSP-amine was 40%. Radiochemical purity was > 98% as determined by TLC and TLE. The inclusion of the thioglycolate reduction step almost doubled the [^{35}S]DMSP-amine yield compared to the procedure described previously (Kocsis *et al.*, 1998).

Enzyme Extraction

Tissue was pulverized in liquid N_2 and thawed in extraction buffer (2 mL g^{-1} fresh weight). Unless otherwise stated, the extraction buffer for SDC (final pH 7.2) was 50 mM potassium-phosphate, 5 mM DL-dithiothreitol, 1 mM Na_2EDTA, 0.1 mM PLP, and 5 mM L-ascorbic acid. The extraction buffer for DOX was the same except that the pH was 8.0 and PLP was omitted. Subsequent steps were at 4°C. The homogenate was centrifuged (10,000*g* for 10 min), and the supernatant was desalted using PD-10 columns (Amersham-Pharmacia Biotech, Uppsala, Sweden) that were equilibrated and eluted with extraction buffer for SDC, or with extraction buffer minus ascorbate (desalting buffer) for DOX. The desalted extracts were clarified by centrifugation (16,000*g* for 5 min) and concentrated about 10-fold with Centricon-30 units (Amicon, Beverly, MA). In some cases samples were then frozen in liquid N_2 and stored at -80°C, which did not affect enzyme activity. Protein was estimated by the Bradford (1976) method using bovine serum albumin as standard.

Enzyme Assays

Assays were carried out at 23 to 25°C. Radiochemical assays were agitated gently on a rotary shaker. No spontaneous breakdown of labeled substrates occurred during the assays. Unless otherwise noted, SDC was assayed in extraction buffer (pH 7.2) with 36

to 50 μM L-[1-^{14}C]SMM using 25-μL reactions and a 75-min incubation time. Assays were carried out in 12- x 75-mm glass tubes closed by a rubber serum stopper to which a CO_2 trap (a 1-cm Whatman No. 3 paper disk containing 20 μL of 2 N KOH) was attached with a pin. Reactions were stopped by injecting 100 μL 10% (w/v) trichloroacetic acid, and incubated for 1 h to maximize transfer of $^{14}CO_2$ to the trap. The trapped $^{14}CO_2$ was quantified by scintillation counting after letting chemiluminescence subside. The trapping efficiency was determined to be 98% using [^{14}C]formate and formate dehydrogenase (Sigma F-5632). The SDC activity data were corrected accordingly. The activity of SDC in potassium-phosphate buffer was twice that in bis(2-hydroxyethyl)iminotris-(hydroxy-methyl)-methane-HCl (Bis-Tris-HCl), *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic) acid-KOH (HEPES-KOH), or 3-(*N*-morpholino)propanesulfonic acid-KOH (MOPS-KOH).

Except where noted, DOX was assayed in desalting buffer (pH 8.0) containing 1 mM [^{35}S]DMSP-amine (2-6 kBq) and 1 to 2 x 10^3 units of bovine liver catalase (Sigma C-40) per assay. The reaction volume was 25 μL in a system like that used for SDC assays, except that the stopper was Teflon-lined and the paper disk contained 20 μL of 30% (w/w) H_2O_2 to trap [^{35}S]DMS. Routine assays were run for 75 min, stopped by injecting 25 μL of 17% (w/v) NaOH, and incubated for 1 h to maximize breakdown of reaction products to [^{35}S]DMS and transfer of [^{35}S]DMS to the trap. The ^{35}S was quantified by scintillation counting. Trap efficiency was determined to be 97% using base-mediated decomposition of [^{35}S]DMSP as the [^{35}S]DMS source (White, 1982; Reed, 1983). The DOX activity data were corrected accordingly. To analyze the labeled products of the assay prior to their decomposition by NaOH, reaction mixtures were

fractionated as described below. To remove O_2, 100 mM β-D-(+)-glucose plus glucose oxidase (Sigma G-9010, 500 units per assay) were added and catalase was omitted. The DOX activity was at least as high in potassium-phosphate buffer as in other buffers tested (the ranking was potassium-phosphate = HEPES-KOH > 1,3-bis[tris(hydroxymethyl) -methylamino]-propane-HCl [Bis-Tris-propane-HCl] > Bis-Tris-HCl).

Catalase assays (final volume 1 mL) contained 65 mM potassium-phosphate (pH 7.0), 0.036% (w/v) H_2O_2, and 1 μL of extract. The reaction was monitored by the decrease in A_{240}. Malate dehydrogenase (MDH) assays (0.8 mL) contained 0.1 M Tris -acetate (pH 8.0), 0.2 mM NADH, 2.5 mM oxaloacetate, and 1 μL of extract. Oxaloacetate-dependent NADH oxidation was measured by the fall in A_{340}.

Stoichiometry of DMSP-amine:CO_2 Production in the SDC Assay

To measure both DMSP-amine and CO_2 formation, L-[U-^{14}C]SMM was used as substrate, $^{14}CO_2$ was trapped as above, and [^{14}C]DMSP-amine was isolated and quantified as follows. After adding unlabeled DMSP-amine and SMM carriers (0.1 μmol each), reaction mixtures were fractionated using 1-mL AG-1 (OH^-) and BioRex-70 (H^+) columns arranged in series. Both columns were washed with water. Then DMSP-amine and SMM were eluted from the BioRex-70 column with 5 mL of 1 N HCl, and the eluate was lyophilized. The DMSP-amine was separated from SMM using TLC system 1 (James *et al.*, 1995b), detected by autoradiography, and quantified by scintillation counting. The recovery of DMSP-amine was determined to be 61% by spiking unlabeled reaction mixtures with [^{35}S]DMSP-amine. This value was used to correct [^{14}C]DMSP -amine data.

Analysis of Labeled Products in the DOX Assay

To analyze DMSO, DMSP, and DMSP-amine, DOX reaction mixtures (not treated with NaOH) were mixed with DMSP, DMSO, and DMSP-amine carriers (0.2-1 μmol) and fractionated on 1-mL columns of AG-1 (OH^-), BioRex-70 (H^+), and AG-50 (H^+) arranged in series. Each column series was washed with 10 mL of water. The effluent contained DMSO. The DMSP-amine was eluted from BioRex-70 with 5 mL of 1 N HCl, and DMSP was eluted from AG-50 with 5 mL of 2.5 N HCl. Samples of the effluent and eluates were counted. When only [^{35}S]DMSP and [^{35}S]DMS were analyzed, a 1-mL mixed resin (AG-1 [OH^-]:BioRex-70 [H^+], 2:1, v/v) column replaced the corresponding separate columns. Recoveries were determined by spiking unlabeled reactions with [^{35}S]DMSP, [^{35}S]DMSO, or [^{35}S]DMSP-amine, and experimental data were corrected accordingly. The identity of [^{35}S]DMSP was authenticated using TLC system 1 and TLE system 1 of James *et al.* (1995b).

Results

Extraction and Assay of SDC Activity

Amino acid decarboxylases can be conveniently assayed by measuring the release of $^{14}CO_2$ from ^{14}C-carboxyl-labeled substrate. However, such assays must first be validated by confirming that CO_2 and amine production are in a 1:1 molar ratio because other reactions in plant extracts may lead to loss of the carboxyl group as CO_2 (Birecka *et al.*, 1985). To test this, L-[U-^{14}C]SMM was used as substrate, and [^{14}C]DMSP-amine and $^{14}CO_2$ were quantified. The formation of [^{14}C]DMSP-amine was readily detected (Figure 19A), and ^{14}C quantification indicated a CO_2:DMSP-amine molar ratio of 0.97 ± 0.01 (mean ± SE; n = 3). Therefore SDC was assayed in subsequent work by measuring

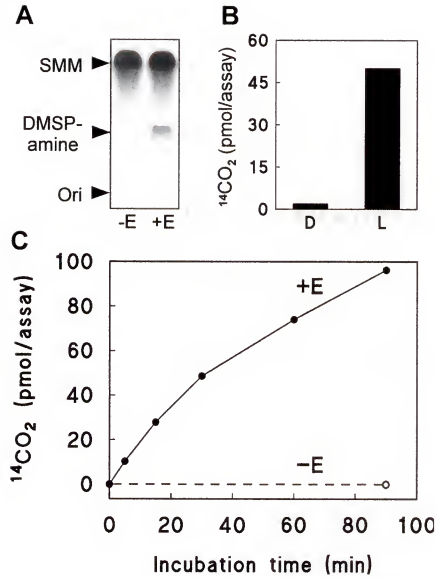

Figure 19. Characterization of SDC activity in *S. alterniflora* leaf extracts. Buffers contained 0.1 mM PLP. A, Formation of [^{14}C]DMSP-amine from L-[U-^{14}C]SMM. L-[U-^{14}C]SMM (1.12 nmol, 10.7 kBq) was incubated with (+E) or without (-E) extract (860 μg protein) for 75 min. The assay mixtures were separated using TLC system 1, and ^{14}C was detected by autoradiography. The positions of standards and the origin (ori) are marked. B, SDC activities assayed using D- or L-[1-^{14}C]SMM as substrate. Assays contained 520 μg protein. Data are means of triplicates. SE values were ≤4% of the means. C, Progress of $^{14}CO_2$ release from L-[1-^{14}C]SMM, plus or minus extract (330 μg protein). Data points are means of triplicates. SE values were ≤4% of the means.

$^{14}CO_2$ release from L-[1-^{14}C]SMM. The SDC activity showed a strong preference for the L-enantiomer of SMM. Using D-[1-^{14}C]SMM gave < 4% of the activity observed with L-[1-^{14}C]SMM (Figure 19B).

Most amino acid decarboxylases have a PLP coenzyme, but a few use a covalently bound pyruvate as prosthetic group instead (Recsei and Snell, 1984; John, 1995). The effect of including PLP (0.1 or 1 mM) in the buffers used to extract and assay SDC was therefore tested. While adding PLP to the assay buffer doubled the activity, adding it to both extraction and assay buffer increased SDC activity 18-fold. These data indicate that SDC requires PLP for stability as well as for activity, and for this reason PLP (0.1 mM) was added to all SDC buffers. Progress curves showed that the rate of $^{14}CO_2$ production from L-[1-^{14}C]SMM declined slowly with time (Figure 19C). This was not due to substrate depletion or product inhibition (see below), and presumably not to abortive transamination (and hence, inactivation) of the coenzyme because PLP was present to replace the inactivated species (John, 1995). Activity loss can therefore be attributed to inactivation of the enzyme itself. A 75-min incubation time was adopted for subsequent work. Using this incubation time, $^{14}CO_2$ formation was in approximate terms linearly related to enzyme level (not shown).

Characteristics of SDC Activity

The SDC showed a broad pH optimum around 7, retaining $\geq$ 75% of the maximum activity between pH 6.5 and 8. The SDC activity was half-maximal at about 18 mM SMM, and its V_{max} was estimated as 0.28 nmol min^{-1} mg^{-1} protein (crude extract). Unlike some other decarboxylases (Grossfeld *et al.*, 1984), SDC is not highly sensitive to inhibition by the amine product. A two-fold molar excess of DMSP-amine over SMM had no effect on activity. The SDC activity appears not to be a side reaction mediated by

one of the decarboxylases found in all plants. This was shown by assaying SDC activity in the presence of a 20-fold molar excess of five L-amino acids for which specific decarboxylases are known (Stevenson *et al.*, 1990; Kumar *et al.*, 1997), all except one (Glu) being structural analogs of SMM (Table XI). None of these compounds caused the drastic inhibition that would be expected were SMM a poor alternative substrate for their respective decarboxylases. In fact, Met, AdoMet, Arg and Glu gave ≤ 18% inhibition, whereas ornithine (Orn) inhibited activity by 60%.

Table XI. Effect of L-amino acids on decarboxylation of L-[1-^{14}C]SMM by *S. alterniflora* extract.
Assays contained 50 μM L-[1-^{14}C]SMM, 1 mM unlabeled L-amino acid, and desalted leaf extract corresponding to 520 μg of protein. The L-amino acids were neutralized with KOH or HCl. Data are the means of duplicates. SE values were ≤1% of the means.

Amino Acid Added	Inhibition
	%
None	0[a]
Met	0
AdoMet	18
Arg	9
Orn	60
Glu	7

[a]The production of $^{14}CO_2$ in the no-addition control was 48.9 pmol per assay.

The SDC Activity of Grasses that Do Not Accumulate DMSP

Comparative biochemistry provided further evidence that the SDC activity is due to an enzyme specific to DMSP synthesis. The SDC activity in *S. alterniflora* was compared with its activity in three other grasses, one of which is another *Spartina* species, that contain very little or no DMSP (Paquet *et al.*, 1994; Kocsis *et al.*, 1998). The house-keeping enzymes, catalase and MDH, were also assayed as controls for the quality of the extracts. Their activities were fairly similar in all species (Table XII). In contrast, only *S. alterniflora* had high SDC activity. The *S. patens* showed 30- to 40-fold less activity, and neither maize nor wheat had detectable activity (Table XII).

Table XII. SDC activity in leaf extracts of various grasses.
SDC activity was measured in desalted leaf extracts of *S. alterniflora* and three grasses that do not accumulate DMSP, *S. patens*, maize, and wheat. SDC was assayed using an L-[1-^{14}C]SMM concentration of 36 μM and a 75 -min incubation time. Catalase and MDH were assayed spectrophotometrically. The experiment was repeated, with similar results.

Species	SDC Activity[a]	Housekeeping Enzyme Activity	
		Catalase	MDH
	pmol h^{-1} mg^{-1} protein	*μmol min^{-1} mg^{-1} protein*	
S. alterniflora	23.9	55.4	10.5
S. patens	0.8	49.5	16.1
Maize	< 0.3	40.5	10.2
Wheat	< 0.2	188	7.4

[a]Assay data were converted to units of pmol h^{-1} mg^{-1} protein by assuming a constant $^{14}CO_2$ production rate. Because the rate declines slowly with time (Figure 19C), this slightly underestimates activity.

Assay for the Conversion of DMSP-amine to DMSP-ald

Because DMSP-amine could, *a priori*, be converted to DMSP-ald by a trans-aminase, dehydrogenase, or oxidase, a radioassay able to measure any of these activities was developed. The principles of the assay are schematized in Figure 20 and can be briefly stated as follows: (a) [^{35}S]DMSP-amine is used as substrate, and when the [^{35}S]DMSP-ald is formed it decomposes rapidly and spontaneously to give [^{35}S]DMS (Trossat *et al.*, 1996); (b) the [^{35}S]DMS is trapped in 30% (w/w) H_2O_2, which oxidizes it to non-volatile [^{35}S]DMSO; (c) as some of the [^{35}S]DMSP-ald formed may be chemically or enzymatically oxidized to [^{35}S]DMSP, [^{35}S]DMSP formation is measured at the end of the assay by fractionating the reaction mixture or by decomposing the [^{35}S]DMSP to [^{35}S]DMS by injecting cold NaOH; and (d) catalase is added to the assay to destroy H_2O_2 generated by DOX activity, or diffusing from the DMS trap.

The assay was tested first with purified porcine kidney diamine oxidase, for which DMSP-amine is a substrate (Bardsley *et al.*, 1971). After a 75-min incubation, that was not followed by NaOH treatment, 19.6% of the [^{35}S]DMSP-amine was converted to [^{35}S]DMS (in the trap), 2.1% to [^{35}S]DMSP, and 0.06% to [^{35}S]DMSO (in the reaction mixture). These data show that [^{35}S]DMSP formation can be significant, and that little oxidation of [^{35}S]DMS to [^{35}S]DMSO occurs in the reaction mixture. This is important because DMSO is not volatile and would not be transferred to the trap.

Evidence for a DOX in *S. alterniflora* Extracts

Desalted *S. alterniflora* extracts gave high rates of DMSP-amine → DMSP-ald conversion in the above assay. The activity was not stimulated by α-keto acids or PLP (Table XIII), indicating that it is not due to a transaminase. Nor was activity increased by adding flavins (Table XIII) or pyridine nucleotides (Figure 21), making a dehydrogenase

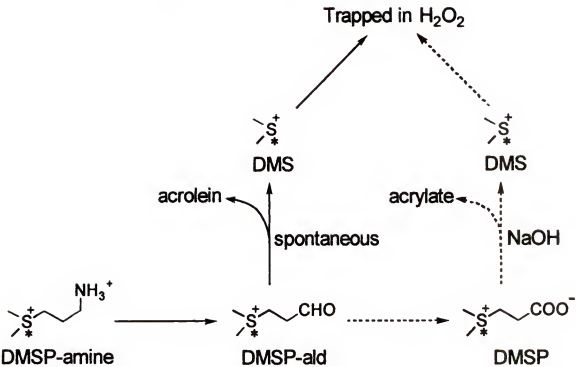

Figure 20. Assay for the enzyme-catalyzed conversion of [^{35}S]DMSP-amine to [^{35}S]DMSP-ald.
The assay was carried out in potassium-phosphate buffer, pH 8, in which [^{35}S]DMSP-ald decomposes spontaneously to acrolein and [^{35}S]DMS with a half-life of <4 min (Trossat *et al.*, 1996). The [^{35}S]DMS released partitions into the gas phase and is trapped on a filter disk soaked in 30% H_2O_2. The H_2O_2 oxidizes [^{35}S]DMS to [^{35}S]DMSO, which is non-volatile. In certain conditions, some [^{35}S]DMSP-ald may be oxidized to [^{35}S]DMSP before it breaks down. At the end of the assay, [^{35}S]DMSP can be decomposed to acrylate and [^{35}S]DMS by treatment with NaOH. *, ^{35}S radiolabel.

Table XIII. The effects of α-keto acids, PLP, or flavins on the conversion of [^{35}S]DMSP -amine to [^{35}S]DMSP-ald by *S. alterniflora* leaf extract. Assays contained 1 mM [^{35}S]DMSP-amine and desalted extract (78 μg of protein). The concentration of α-keto acids, FMN, and FAD was 1 mM. The PLP concentration was 0.1 mM. Incubation was for 75 min. The conversion of [^{35}S]DMSP-amine to [^{35}S]DMSP-ald was estimated from total [^{35}S]DMS production when base was added after incubation. Values are the means of duplicate assays. SE values were ≤2% of the means.

Addition	Activity	
	pmol/assay	
None	924	(100)[a]
α-Ketoglutarate	940	(101)
Oxaloacetate	796	(86)
Pyruvate	897	(97)
Glyoxylate	887	(99)
β-Hydroxypyruvate	849	(95)
PLP	891	(97)
FMN	813	(88)
FAD	870	(94)

[a]Values in parentheses are activities as percent of the no-addition control.

unlikely. Although NAD and NADP did not increase activity, they caused a switch in the major reaction product from [^{35}S]DMS to [^{35}S]DMSP (Figure 21). This is anticipated because *S. alterniflora* is expected to possess NAD(P)-linked DMSP-ald dehydrogenase activity (Kocsis *et al.*, 1998). Adding NAD(P) as well as DMSP-amine to *S. alterniflora*

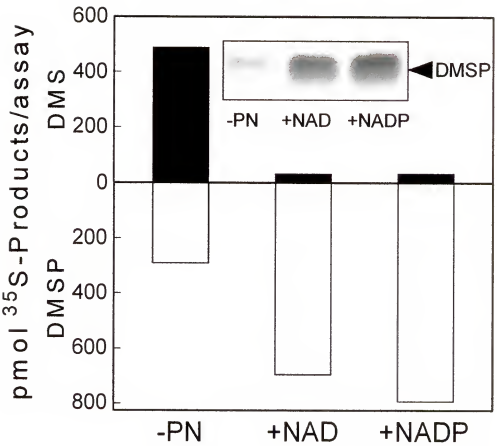

Figure 21. Effect of pyridine nucleotides on conversion of [^{35}S]DMSP-amine to [^{35}S]DMS and [^{35}S]DMSP by *S. alterniflora* leaf extract. After desalting twice, extract (470-720 μg of protein per assay) was incubated for 75 min with 25 nmol (3.7 kBq) of [^{35}S]DMSP-amine, minus (-PN) or plus 1 mM NAD or NADP. L-Ascorbate (5 mM) was included in the assay buffer. Data are means of two or three replicates, normalized to 500 μg of protein per assay. SE values were ≤12% of the means. The identity of the [^{35}S]DMSP formed was confirmed by TLC and autoradiography (inset).

extracts therefore reconstitutes the last two steps in the DMSP pathway (Figure 18). The above results suggest, by elimination, that the enzyme mediating the DMSP-amine → DMSP-ald conversion is an oxidase. Direct evidence for this was obtained by removing O_2 from the assay using glucose oxidase plus glucose (Table XIV). The glucose oxidase/

glucose system reduced activity by 96% when used alone, and by 99% when combined with a nitrogen atmosphere.

Table XIV. The O_2-dependence of the conversion of [^{35}S]DMSP-amine to [^{35}S]DMSP -ald by *S. alterniflora* leaf extract.
Assays contained 1 mM [^{35}S]DMSP-amine and desalted extract (75 μg of protein), and were carried out in air except where indicated. The final concentration of glucose (Glc) was 100 mM. Glucose oxidase (GOX) was added at 500 units per assay. Catalase was omitted so that H_2O_2 generated by GOX would not be reconverted to O_2. The conversion of [^{35}S]DMSP -amine to [^{35}S]DMSP-ald was estimated from total [^{35}S]DMS production when base was added after incubation. Subsequent fractionation of reaction mixtures confirmed that no [^{35}S]DMSO was formed. Values are the means of duplicate assays. SE values were ≤3% of the means.

Addition	Activity	
	pmol/assay	
None	730	(100)[a]
Glc	725	(100)
GOX	675	(93)
Glc/GOX	30	(4)
Glc/GOX (N_2)[b]	5	(1)

[a]Values in parentheses are activities as percent of the no-addition control.
[b]The head space of the reaction was flushed with N_2 for 1 min before starting the assay.

Characteristics of DOX Activity

Fractionation of assay mixtures showed that [^{35}S]DMSP became a major product as the amount of extract increased, but that [^{35}S]DMSO formation was never very important (Figure 22A). The relationship between [^{35}S]DMSP formation and extract

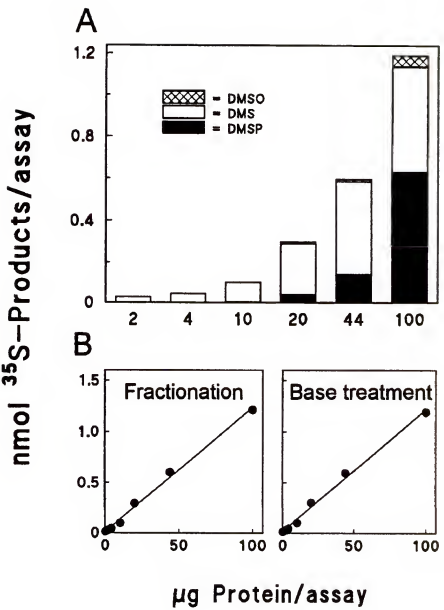

Figure 22. Effect of *S. alterniflora* extract concentration on the nature and amounts of the products formed in the DOX assay.
Assays containing 1 mM [^{35}S]DMSP-amine and extract equivalent to 2 to 100 µg of protein were incubated for 75 min, and then either fractionated as described in "Materials and Methods" or treated with 25 µL 17% NaOH to decompose [^{35}S]DMSP to [^{35}S]DMS. A, Proportions of [^{35}S]DMS, [^{35}S]DMSP, and [^{35}S]DMSO formed as a function of the amount of protein per assay. B, Relationship between the amount of protein per assay and either the summed ^{35}S-products obtained by fractionation ([^{35}S]DMS + [^{35}S]DMSP + [^{35}S]DMSO) (left) or the total amount of [^{35}S]DMS formed when assays were treated with base (right). Data are means of two to five replicates. The sum of the labeled reaction products, plus remaining [^{35}S]DMSP-amine, equaled the amount of [^{35}S]DMSP-amine substrate added to the assay.

concentration is presumably due mainly to DMSP-ald dehydrogenase activity, supported by traces of NAD(P) left after desalting (compare with Figure 21) and by NAD(P)H oxidase activity in the extract. Total [^{35}S]DMSP-ald formation, whether estimated from the sum of labeled products after fractionation or from total [^{35}S]DMS production when assays were treated with base, was almost the same and was linearly related to the amount of protein (Figure 22B). The base-treatment procedure, which is simpler, was therefore adopted for routine use. Formation of [^{35}S]DMSP-ald was linear with time for 2 h (not shown). The DOX activity was maximal at pH 7.5 to 8, and showed a V_{max} of 0.37 nmol min^{-1} mg^{-1} protein (crude extract), and an apparent K_m for DMSP-amine of 1.8 mM. Addition of L-ascorbate (5 mM) inhibited activity, but improved enzyme extraction. It was therefore routinely added to the DOX extraction buffer and removed by desalting.

Because DMSP-amine is structurally similar to diamines and polyamines, the selectivity of DOX was investigated by adding a 5- or 20-fold molar excess of unlabeled di- and polyamines to DOX assays (Figure 23). The polyamines spermidine and spermine had little effect, but the diamines 1,3-diaminopropane, putrescine, and cadaverine reduced DOX activity by 90-98% when present in 20-fold excess. This shows that DOX is not highly selective for DMSP-amine, and suggests that it may be related to diamine oxidases. Because these are copper-containing enzymes (Smith, 1985), an attempt was made to remove enzyme-bound copper using 10 mM diethyldithiocarbamate or 200 mM EDTA. These treatments gave preparations that were stimulated 20 to 30% by 1 mM $CuSO_4$, which was not the case for untreated controls. As copper is hard to strip out of some amine oxidases (Hysmith and Boor, 1988), this result is not inconsistent with a copper requirement.

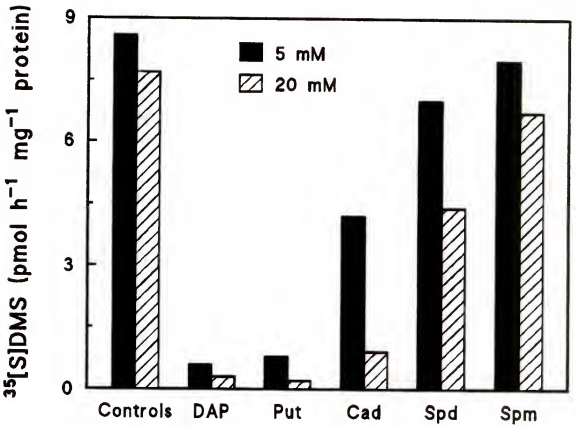

Figure 23. Effect of diamines and polyamines on DOX activity in *S. alterniflora* leaf extract.
Assays contained 1 mM [^{35}S]DMSP-amine, and either 5 or 20 mM unlabeled amine or an equivalent quantity of KCl (controls). The reaction mixtures were treated with NaOH to convert [^{35}S]DMSP to [^{35}S]DMS. Data are means of duplicate assays. SE values were ≤2% of the means. DAP, 1,3-diamino-propane; Put, putrescine; Cad, cadaverine; Spd, spermidine; Spm, spermine.

The DOX Activity of Grasses that Do Not Accumulate DMSP

The activities of DOX, catalase, and MDH were measured in *S. alterniflora* and in the non-DMSP-accumulating species *S. patens*, maize, and wheat (Table XV). The DOX activity in *S. alterniflora* was about 100-fold higher than in the other plants, whereas catalase and MDH activities were quite similar in all species. These data indicate that the

DOX activity in *S. alterniflora* is due principally to an enzyme specific to DMSP synthesis and not to an oxidase of widespread occurrence.

Table XV. DOX activity in leaf extracts of various grasses.
DOX activity was measured in extracts of *S. alterniflora* and three grasses that do not accumulate DMSP. DOX was assayed with a [^{35}S]DMSP-amine concentration of 1 mM and a 75-min incubation time using total [^{35}S]DMS production when base was added after incubation. Catalase and MDH were assayed spectrophotometrically. Data are means of duplicates. SE values were ≤12% of the means.

Species	DOX Activity	Housekeeping Enzyme Activity	
		Catalase	MDH
	pmol h^{-1} mg^{-1} protein	*μmol min^{-1} mg^{-1} protein*	
S. alterniflora	10,800	180	16.8
S. patens	145	85	16.7
Maize	44	68	12.2
Wheat	157	235	10.0

Discussion

As a result of this work, novel enzyme activities have been identified in *S. alterniflora* that catalyze the two steps unique to DMSP synthesis in this species, namely the decarboxylation of SMM and the oxidation of DMSP-amine to DMSP-ald. These enzymes are SDC and DOX. Also, convenient radioassays for SDC and DOX were devised here, along with an improved procedure for synthesizing the [^{35}S]DMSP-amine substrate required for the DOX assay. The SDC and DOX activities were shown to be

robust inasmuch as they withstand freeze-thaw treatment, desalting, and concentration. Together, these advances open the way for future work to purify and characterize SDC and DOX, and to clone their cDNAs.

The *in-vivo* flux through the pathway SMM → DMSP-amine → DMSP-ald → DMSP was estimated from computer modeling of radiotracer data to be 1.6 ± 0.7 nmol min^{-1} g^{-1} fresh weight (Kocsis *et al.*, 1998). The V_{max} values for SDC and DOX activities in *S. alterniflora* extracts (0.28 and 0.37 min^{-1} mg^{-1} protein, respectively) are adequate to account for this flux, because leaf protein content in *S. alterniflora* is about 10 mg g^{-1} fresh weight. Modeling of the *in vivo* labeling data also predicted that the K_m for SMM decarboxylation would be an order of magnitude higher than that for DMSP-amine oxidation (310 versus 5.8 nmol g^{-1} fresh weight, each value being subject to an error of about ± 50%) (Kocsis *et al.*, 1998). This prediction agrees well with the 10-fold difference between the apparent K_m values that were estimated for the substrates of SDC (about 18 mM) and DOX (1.8 mM).

The absence or very low level of SDC activity in grasses that do not accumulate DMSP indicates that SDC is an enzyme specific to the DMSP pathway. The relative insensitivity of SDC to inhibition by L-amino acids reinforces this inference by showing that SDC activity is unlikely to be a side-reaction mediated by the ubiquitous decarboxylases whose physiological substrates are Met, AdoMet, Arg, Orn, or Glu. The lack of AdoMet inhibition also suggests that SDC is not closely related to AdoMet decarboxylase, even though SMM is very similar in structure to AdoMet. Additional evidence that SDC and AdoMet decarboxylase are unrelated is that SDC requires PLP, whereas AdoMet decarboxylase belongs to the small group of enzymes that use a catalytic

pyruvoyl residue, not PLP (Xiong *et al.*, 1997). On the other hand, the modest inhibition of SDC by L-Orn suggests a possible relationship to Orn decarboxylase. Inasmuch as eukaryotic Orn decarboxylases and other basic amino acid decarboxylases share amino acid sequence homology (Sandmeier *et al.*, 1994), such a relationship could offer an indirect approach to the cDNA cloning of SDC.

The very low DOX activities in grasses that lack DMSP imply that DOX is an enzyme associated specifically with DMSP synthesis. The trace of activity found in these species may be because plant diamine oxidases have some activity towards DMSP -amine, as has been demonstrated for the porcine kidney enzyme (Bardsley *et al.*, 1971). Whatever the nature of the slight DOX activity in species that do not accumulate DMSP, this activity is consistent with the finding that such species have a low capacity to oxidize exogenously supplied DMSP-amine (Kocsis *et al.*, 1998). Assuming DOX to be a specific enzyme, it might *a priori* be related to diamine oxidases or to polyamine oxidases because both types of enzymes occur in grasses (Smith, 1985; Suzuki and Hagiwara, 1993), and DMSP-amine can be considered to be a diamine or polyamine analog. Diamine oxidases and polyamine oxidases are quite different, the former being copper enzymes with a covalently bound topaquinone cofactor and the latter being flavin -containing enzymes (Smith, 1985; Klinman, 1996). That DOX is far more sensitive to inhibition by diamines than polyamines, and modestly stimulated by copper after chelation treatment, suggests that it is more likely to be a member of the copper amine oxidase family. The substantial sequence identity among the members of this family (Padiglia *et al.*, 1998) may, as for SDC, permit the cDNA cloning of DOX by a homology-based approach.

CHAPTER 6
CONCLUSIONS

The long-term goal of this study is to further the metabolic engineering of abiotic stress-resistance in crop plants. The approach taken here was to elucidate how the saltmarsh grass, *Spartina alterniflora*, makes the compatible solute 3-dimethylsulfonio-propionate (DMSP) from L-Met, and how the DMSP pathway fits with primary plant sulfur metabolism. This has been done as a first step towards acquiring the necessary understanding of the metabolic pathway, and towards cloning the genes encoding the enzymes that catalyze the pathway reactions.

Accumulated DMSP is known to function in osmoprotection and cryoprotection, and it likely also plays a role in resistance to oxidative stress. The accumulation of DMSP is a rational target for metabolic engineering because, by definition, the cellular concentrations of DMSP needed to be effective should not hinder normal cellular function. Additionally, DMSP is a betaine analog that contains sulfur instead of nitrogen, and is thus attractive from an agricultural standpoint since DMSP accumulation would not result in an increased demand for nitrogen. Also attractive from an agricultural standpoint is elucidation of the DMSP pathway in a grass. The world's five most important crop plants are grasses (Evans, 1993), and the additional understanding of sulfur metabolism in grasses along with insight gained by using non-grass models, and the potential for isolating grass genes, will be very helpful in advancing subsequent steps in the engineering process.

Many phylogenetically diverse marine algae appear to make DMSP from L-Met via intermediates that are not found in common with DMSP synthesis in the dicot, *Wollastonia biflora* (Figure 24). Of the intermediates found in the pathway of *W. biflora*, *S*-methyl-Met (SMM) is the most interesting because it has recently been shown to be a ubiquitous angiosperm metabolite. In this study, the DMSP synthesis pathway of *S. alterniflora* was shown to differ from the algal and dicot pathways; however, in both *S. alterniflora* and *W. biflora* DMSP synthesis extends from SMM (Figure 24). Thus, in angiosperms, L-Met is converted to SMM via Met *S*-methyltransferase (MMT), which constitutes half of the apparently futile SMM cycle, and the different DMSP pathways of angiosperms branch from the SMM cycle. In addition to the AdoMet-dependent methylation mediated by MMT, the SMM cycle involves the reconversion of SMM to Met by homocysteine *S*-methyltransferase (HMT).

Previous work has shown that SMM is most probably ubiquitous in angiosperms, that MMT and HMT genes are expressed in all organs of Arabidopsis, and that the SMM cycle is active throughout the plant. Thus, SMM is a primary plant metabolite. Although various hypotheses had been advanced to account for the presence of SMM or its cycle, there was little experimental support for any of them. In the first phase of this work, MMT knockouts in Arabidopsis and maize were therefore sought and used to investigate the importance of SMM and its cycle.

The MMT knockout experiments show that the hypotheses that SMM is an essential methyl donor in plants, or that it is a necessary transport form of sulfur, can be

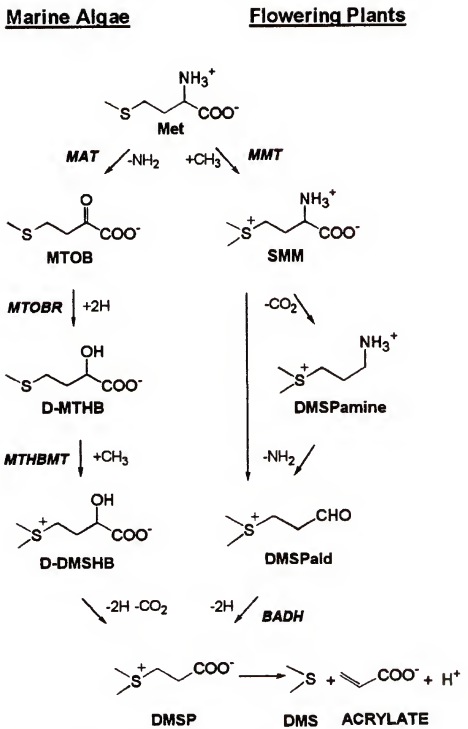

Figure 24. Biosynthetic pathways of DMSP in marine algae and flowering plants. The algal pathway has been found in the green macroalga *E. intestinalis* and various plankton species. Two related pathways have been found in flowering plants, one in the saltmarsh grass *S. alterniflora* (DMSP-amine intermediate) and one in the Pacific coastal dicot *W. biflora*. Marine algae have a DMSP lyase that can cleave DMSP to give DMS and acrylate. MAT, Met aminotransferase; MTOBR, MTOB reductase; MTHBMT, MTHB methyltransferase.

ruled out. Eliminating MMT, and hence SMM and the SMM cycle, has no appreciable effect on plant growth and development in favorable culture conditions. The SMM cycle has been hypothesized to stop depletion of L-Met pools caused by excess AdoMet synthesis. Additionally, the SMM cycle has been hypothesized to govern AdoMet level and hence AdoMet/ AdoHcy (methylation) ratio. Previous *in silico* modeling suggested the second hypothesis explaining the existence of the SMM cycle to be correct. Computer modeling of data obtained for wild-type Arabidopsis leaves indicated a 160% increase in AdoMet level when the SMM cycle was eliminated, while free Met levels did not change. Additionally, the free Met pool recovered from a simulated overshoot with or without the SMM cycle. The results obtained with *mmt* knockouts of Arabidopsis in this study generally agree with the model. Free Met did not change and AdoMet increased, although only by 15%. Additionally, AdoHcy levels went down in the *mmt* knockouts, so that the methylation ratio in leaves was increased by 45%. Thus, it appears that the SMM cycle has a role in controlling the methylation ratio. This work therefore assigns a function to the SMM cycle for the first time. Moreover, considering the limitations of the computer model, it for all intents and purposes correctly predicted the function of the SMM cycle and underscores the need for such modeling in any rational attempt at metabolic engineering.

In the second phase of this work, *in vivo* radio- and stable-isotope feeding studies showed that the DMSP synthesis pathway in *S. alterniflora* is different from the pathways known from *W. biflora* or marine algae, although, like the *W. biflora* pathway, its starting point is SMM. Overall, the DMSP pathway in *S. alterniflora* proceeds as follows: L-Met

→ SMM → 3-dimethylsulfoniopropylamine (DMSP-amine) → 3-dimethylsulfoniopropionaldehyde (DMSP-ald) → DMSP. In distinction to the *W. biflora* pathway, the *S. alterniflora* pathway involves DMSP-amine as an intermediate. This is the first time DMSP-amine has been reported as an endogenous metabolite in any organism. Also, this work indicates that the DMSP pathway has evolved at least three times.

Although all the known DMSP pathways start with the primary precursor, L-Met, and result in the formation of DMSP, the angiosperm pathways have no intermediates in common with the algal pathway. The angiosperm pathways, however, have in common the intermediates SMM and DMSP-ald, but they differ with respect to how they convert SMM to DMSP-ald. This shows that not only did DMSP synthesis evolve independently in the ancestors of algae and higher plants, but that the ability to make DMSP evolved at least twice in angiosperms. In *S. alterniflora*, SMM is first decarboxylated to form DMSP-amine, which is then in turn converted to DMSP-ald (Kocsis *et al.*, 1998). In *W. biflora*, it appears that the one-step mechanism by which SMM is converted directly to DMSP-ald involves either a transaminase-decarboxylase enzyme complex or an unusual enzyme that can carry out both the necessary steps (Rhodes *et al.*, 1997). In *W. biflora*, the enzyme that catalyzes the conversion of DMSP-ald to DMSP, DMSP-ald dehydrogenase (DDH), and betaine aldehyde dehydrogenase (BADH) are apparently one and the same (Trossat *et al.*, 1997; Vojtěchová *et al.*, 1997). In *S. alterniflora*, as in *W. biflora*, the DMSP pathway co-occurs with the glycine betaine pathway (Rhodes and Hanson, 1993). It is likely, therefore, that *S. alterniflora* has also recruited BADH from the glycine betaine pathway to catalyze the last step in the synthesis of DMSP.

Finally, an approach based on comparative biochemistry showed that the *S. alterniflora* enzyme activities responsible for both the decarboxylation of SMM and the conversion of DMSP-amine to DMSP-ald are unique to the biosynthesis of DMSP in this grass. When labeled SMM was fed to *S. alterniflora* and several phylogenetically diverse grasses, including the closely related non-DMSP-accumulating grass, *S. patens*, none except *S. alterniflora* showed label accumulating as DMSP-amine or DMSP. This was in spite of the fact that all the grasses readily took up the labeled SMM. Additionally, tracer and substrate-level doses of labeled DMSP-amine were fed to a diverse range of grass species. Again, in spite of label being readily taken up, only *S. alterniflora* accumulated significant amounts of labeled DMSP.

Moreover, *in vitro* enzyme radioassays were developed for measuring SMM decarboxylase (SDC) and the conversion of DMSP-amine to DMSP-ald in leaf extracts. The SDC is critically PLP-dependent and has a broad pH optimum around 7. The enzyme responsible for the conversion of DMSP-amine to DMSP-ald is not a dehydrogenase, nor is it a transaminase, but is in fact an oxidase. The DMSP-amine oxidase (DOX) does not require added FAD or FMN, but requires molecular oxygen for activity. It exhibits optimal activity at pH 7.5 to 8. Both SDC and DOX are novel enzyme activities that have not previously been detected, and are unique to the synthesis of DMSP in *S. alterniflora*. When compared to *S. patens*, maize, and wheat, species that do not accumulate DMSP, only *S. alterniflora* has appreciable SDC or DOX activity. The apparent K_m values for the organic substrates of SDC and DOX are about 18 mM and 1.8 mM, respectively. As predicted by computer modeling of data obtained by feeding radiolabeled L-Met to *S. alterniflora* leaves, and confirmed by comparative biochemistry,

SDC and DOX are the novel enzymes of DMSP synthesis in *S. alterniflora*. The SDC has an apparent affinity for its substrate, SMM, that is an order of magnitude less than the one DOX has for its organic substrate, DMSP-amine, as predicted by the model. The *S. alterniflora* pathway is a good one to engineer, since it is not branched and DMSP is not further metabolized in angiosperms.

The *in vivo* radiotracer and *in vitro* enzyme work on the DMSP pathway in *S. alterniflora* together lay the groundwork for the purification of SDC and DOX, two novel, pathway-specific enzymes whose encoding genes are needed to undertake metabolic engineering of DMSP synthesis in plants that lack it. This represents a new avenue for engineering stress-resistant crops. Having already elucidated the DMSP biosynthesis pathway in *S. alterniflora*, amino acid sequence information from purified SDC and DOX would be used to obtain the encoding cDNAs for future use in metabolically engineering DMSP synthesis in crop plants. In addition, information obtained from such metabolic analyses will further the understanding of sulfur metabolism in higher plants, and may allow for the generation of more complete models of the global sulfur cycle. Since *S. alterniflora* is a C_4 grass (Thompson, 1991), and several aspects of sulfur metabolism in higher plants are known to involve specialized roles for bundle sheath cells compared to mesophyll cells (Burgener *et al.*, 1998; Anderson, 1990; Schmutz and Brunold, 1984), the cellular distribution of the enzymes involved in the DMSP pathway needs to be determined in the leaves of *S. alterniflora* as well. Finally, it seems likely that, as in *W. biflora*, there may be differences in the subcellular distribution of the pathway enzymes in *S. alterniflora*. Movement of DMSP biosynthetic intermediates from the cytosol to the chloroplast stroma, for example, may represent additional constraints on metabolic flux

through the pathway and, therefore, additional challenges to metabolic engineering of the DMSP pathway. This means that for successful engineering to proceed, the subcellular distribution of the pathway enzymes and possible transporters of the pathway intermediates should also be investigated.

APPENDIX
GROWTH AND DEVELOPMENT OF WILD-TYPE VERSUS MUTANT ARABIDOPSIS

The table on the next page summarizes data comparing the growth and development of wild-type versus *mmt* mutant (knockout) Arabidopsis. Compared to wild-type Arabidopsis, mutants had no phenotype, with the exception of seed germination. Germination in light and in the dark was significantly different when seeds were germinated in plastic (polystyrene) Petri dishes on Whatman No. 3 filter paper moistened with distilled water. In the dark, *mmt* mutant seeds had a germination rate of 11.7% versus 3.0% for wild type. In the light, germination rates were 66.3% and 98.0% for wild type and *mmt* mutant, respectively. Immediately prior to germination in a growth cabinet, seeds were stratified for 2 days. Germinants were counted 4 days after placing the seeds in a growth cabinet. Growth cabinet settings were those routinely used to grow Arabidopsis (see CHAPTER 3, **Materials and Methods, Plant Material and Growing Conditions**). During routine growth of Arabidopsis on germinating mix, no difference was observed between *mmt* mutant and wild type germination rates, and for each genotype the germination rate was well over 90% (data not shown).

Table XVI. Summary of growth and development data for homozygous wild-type and homozygous *mmt* mutant (knockout) Arabidopsis.

An unpaired Student's t test was performed for the pair of means in each row representing wild-type versus knockout plants. The standard error and number of replicates (N value) are indicated for each mean. Two-tailed p values of <0.05 were considered to be statistically significant.

Measured Parameter		Mean for Wild Type	Standard Error for Wild Type	N Value for Wild Type	Mean for Knockout	Standard Error for Knockout	N Value for Knockout	Degrees of Freedom	t Value	p Value	Statistically Significant? (Yes/No)
Shoot FW (mg) for:	week 1	0.9	0.2	15	0.8	0.1	15	28	0.4472	0.6582	No
	week 2	2.1	0.4	15	1.8	0.4	15	28	0.5303	0.6001	No
	week 3	43	6	15	44	12	15	28	0.0745	0.9411	No
	week 4	434	71	10	375	50	10	18	0.6794	0.5055	No
	week 5	1,058	140	10	1,217	163	10	18	0.7400	0.4689	No
	week 6	2,168	195	10	2,222	108	10	18	0.2422	0.8113	No
	week 7	2,484	191	10	2,263	125	10	18	0.9682	0.3458	No
Shoot DW (mg) for:	week 3	5	1	15	5	1	15	28	0.0000	1.0000	No
	week 4	54	7	10	47	5	10	18	0.8137	0.4264	No
	week 5	144	17	10	168	22	10	18	0.8632	0.3994	No
	week 6	342	34	10	342	25	10	18	0.0000	1.0000	No
	week 7	541	40	10	489	26	10	18	1.0900	0.2901	No
Root Length (mm) for:	week 1	8	3	5	6	2	5	8	0.5547	0.5943	No
	week 2	11	3	5	9	1	5	8	0.6325	0.5447	No
	week 3	21	2	5	22	5	5	8	0.1857	0.8573	No
Day of First Anthesis		28.9	0.4	20	28.5	0.4	20	38	0.7071	0.4838	No
Shoot DW at Harvest (g)		0.49	0.04	20	0.50	0.04	20	38	0.1768	0.8606	No
Seed Yield (mg)		144	15	20	138	15	20	38	0.2828	0.7788	No
Harvest Index (g/g)		0.29	0.1	20	0.27	0.1	20	38	0.1414	0.8883	No
Seed Germination in Dark (%)		3.0	1.0	3	11.7	1.3	3	4	5.3045	0.0061	Yes
Seed Germination in Light (%)		66.3	1.6	6	98.0	0.4	6	10	19.2209	0.0001	Yes

LIST OF REFERENCES

Abrahamson L, Shapiro SK (1965) The biosynthesis of methionine: partial purification and properties of homocysteine methyltransferase of jack bean meal. Arch Biochem Biophys **109:** 376-382

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1989) Molecular Biology of the Cell, 2nd edn. Garland Publishing, Inc., New York, NY

Amir R, Hacham Y, Galili G (2002) Cystathionine γ-synthase and threonine synthase operate in concert to regulate carbon flow towards methionine in plants. Trends Plant Sci **7:** 153-156.

Anderson JW (1990) Sulfur metabolism in plants. *In* BJ Miflin, PJ Lea, eds, The Biochemistry of Plants: A Comprehensive Treatise, Vol 16: Intermediary Nitrogen Metabolism. Academic Press, San Diego, CA, pp 327-381

Aneja VP, Cooper, WJ (1989) Biogenic sulfur emissions: a review. *In* ES Saltzman, WJ Cooper, eds, Biogenic Sulfur in the Environment. American Chemical Society, Washington, DC, pp 2-13

Appling DR (1991) Compartmentation of folate-mediated one-carbon metabolism in eukaryotes. FASEB J **5:** 2645-2651

Attieh JM, Hanson AD, Saini HS (1995) Purification and characterization of a novel methyltransferase responsible for biosynthesis of halomethanes and methanethiol in *Brassica oleracea*. J Biol Chem **270:** 9250-9257

Awal HMA, Yoshida I, Doe M, Hirasawa E (1995) 3-Aminopropionaldehyde dehydrogenase of millet shoots. Phytochemistry **40:** 393-395

Azevedo RA, Arruda P, Turner WL, Lea PJ (1997) The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochemistry **46:** 395-419

Bardsley WG, Ashford JS, Hill CM (1971) Synthesis and oxidation of aminoalkyl -onium compounds by pig kidney diamine oxidase. Biochem J **122:** 557-567

Bensen RJ, Johal GS, Crane VC, Tossberg JT, Schnable PS, Meeley RB, Briggs SP (1995) Cloning and characterization of the maize *An1* gene. Plant Cell **7:** 75-84

Bergmann L, Rennenberg H (1993) Glutathione metabolism in plants. *In* LJ De Kok, I Stulen, H Rennenberg, C Brunold, WE Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic Publishing, Hague, Netherlands, pp 102-123

Bezzubov AA, Gessler NN (1992) Plant sources of *S*-methylmethionine. Prikl Biokhim Mikrobiol **28:** 423-429

Birecka H, Bitonti AJ, McCann PP (1985) Assaying ornithine and arginine decarboxylase in some plant species. Plant Physiol **79:** 509-514

Blunden G, Gordon SM (1986) Betaines and their sulphonio analogues in marine algae. Prog Phycol Res **4:** 39-80

Bourgis F, Roje S, Nuccio ML, Fisher DB, Tarczynski MC, Li C, Herschbach C, Rennenberg H, Pimenta MJ, Shen TL, Gage DA, Hanson AD (1999) *S*-Methylmethionine plays a major role in phloem sulfur transport and is synthesized by a novel type of methyltransferase. Plant Cell **11:** 1485-1498

Bowyer JR, Leegood RC (1997) Photosynthesis. *In* PM Dey, JB Harborne, eds, Plant Biochemistry. Academic Press, San Diego, CA, pp 49-110

Boyer JS (1982) Plant productivity and environment. Science **218:** 443-448

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248-254

Brinch-Pedersen H, Galili G, Knudsen S, Holm PB (1996) Engineering of the aspartate family biosynthetic pathway in barley (*Hordeum vulgare* L.) by transformation with heterologous genes encoding feed-back-insensitive aspartate kinase and dihydrodipicolinate synthase. Plant Mol Biol **32:** 611-620

Burgener M, Suter M, Jones S, Brunold C (1998) Cyst(e)ine is the transport metabolite of assimilated sulfur from bundle-sheath to mesophyll cells in maize leaves. Plant Physiol **116:** 1315-1322

Campisi L, Yang Y, Yi Y, Heilig E, Herman B, Cassista AJ, Allen DW, Xiang H, Jack T (1999) Generation of enhancer trap lines in Arabidopsis and characterization of expression patterns in the inflorescence. Plant J **17:** 699-707

Cantoni GL (1977) *S*-Adenosylmethionine: present status and future perspectives. *In* F Salvatore, E Borek, HG Williams-Ashman, F Schlenk, eds, The Biochemistry of Adenosylmethionine. Columbia University Press, New York, NY, pp 557-577

Cantoni GL, Richards HH, Chiang PK (1979) Inhibitors of *S*-adenosylhomocysteine hydrolase and their role in the regulation of biological methylation. *In* E Usdin, RT Borchardt, ER Creveling, eds, Transmethylation. Elsevier, New York, NY, pp 155-164

Capdevila A, Wagner C (1998) Measurement of plasma *S*-adenosylmethionine and *S*-adenosylhomocysteine as their fluorescent isoindoles. Anal Biochem **264:** 180-184

Caudill MA, Wang JC, Melnyk S, Pogribny IP, Jernigan S, Collins MD, Santos -Guzman J, Swendseid ME, Cogger EA, James SJ (2001) Intracellular *S*-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl -deficient cystathionine beta-synthase heterozygous mice. J Nutr **131:** 2811-2818

Chae HS, Faure F, Kieber J (2003) The *eto1*, *eto2*, and *eto3* mutations and cytokinin treatment increase ethylene biosynthesis in Arabidopsis by increasing the stability of ACS protein. Plant Cell **15:** 545-559

Chapple CCS, Glover JR, Ellis BE (1990) Purification and characterization of methionine:glyoxylate aminotransferase from *Brassica carinata* and *Brassica napus*. Plant Physiol **94:** 1887-1896

Charlson RJ, Lovelock JE, Andreae MO, Warren SG (1987) Oceanic phytoplankton, atmospheric sulphur, cloud albedo and climate. Nature **326:** 655-661

Colmer TD, Fan TW-M, Läuchli A, Higashi RM (1996) Interactive effects of salinity, nitrogen and sulphur on the organic solutes in *Spartina alterniflora* leaf blades. J Exp Bot **47:** 369-375

Cooper AJL, Hanson AD (1998) Advances in the enzymology of the biogeochemical sulfur cycle. Chemtracts-Biochem Mol Biol **11:** 729-747

Cooper WJ, Matrai PA (1989) Distribution of dimethyl sulfide in the oceans: a review. *In* ES Saltzman, WJ Cooper, eds, Biogenic Sulfur in the Environment. American Chemical Society, Washington, DC, pp 140-151

Cosquer A, Pichereau V, Pocard J-A, Minet J, Cormier M, Bernard T (1999) Nanomolar levels of dimethylsulfoniopropionate, dimethylsulfonioacetate, and glycine betaine are sufficient to confer osmoprotection to *Escherichia coli*. Appl Environ Microbiol **65:** 3304-3311

Cossins EA (1987) Folate biochemistry and the metabolism of one-carbon units. *In* DD Davies, ed, The Biochemistry of Plants: A Comprehensive Treatise, Vol 11: Biochemistry of Metabolism. Academic Press, San Diego, CA, pp 317-353

Cossins EA, Chen L (1997) Folates and one-carbon metabolism in plants and fungi. Phytochemistry **45:** 437-452

Crane PR, Friis EM, Pedersen KR (1995) The origin and early diversification of angiosperms. Nature **374:** 27-33

Curien G, Job D, Douce R, Dumas R (1998) Allosteric activation of *Arabidopsis* threonine synthase by *S*-adenosylmethionine. Biochemistry **37:** 13212-13221

Dacey JWH, King GM, Wakeham, SG (1987) Factors controlling emission of dimethylsulphide from salt marshes. Nature **330:** 643-645

Dellaporta S (1994) Plant DNA miniprep and microprep: versions 2.1-2.3. *In* M Freeling, V Walbot, eds, The Maize Handbook. Springer-Verlag, New York, NY, pp 522-525

Dickson DMJ, Kirst GO (1986) The role of β-dimethylsulphoniopropionate, glycine betaine and homarine in the osmoacclimation of *Platymonas subcordiformis*. Planta **167:** 536-543

Dickson DMJ, Wyn Jones RG, Davenport J (1980) Steady state osmotic adaptation in *Ulva lactuca*. Planta **150:** 158-165

Dodd WA, Cossins EA (1970) Homocysteine-dependent transmethylases catalyzing the synthesis of methionine in germinating pea seeds. Biochim Biophys Acta **201:** 461 -470

Evans LT (1993) Crop Evolution, Adaptation and Yield. Cambridge University Press, Cambridge, UK

Fahey RC, Newton GL (1987) Determination of low-molecular weight thiols using monobromobimane fluorescent labeling and high-performance liquid chromatography. Methods Enzymol **143:** 85-96

Fell DA (1997) Understanding the Control of Metabolism. Portland Press, London, UK

Finley KT, Wilson J Jr (1970) Fundamental Organic Chemistry. Prentice-Hall, Englewood Cliffs, NJ

Fitzgerald MA, Ugalde TD, Anderson JW (2001) Sulphur nutrition affects delivery and metabolism of S in developing endosperms of wheat. J Exp Bot **52:** 1519-1526

Gage DA, Rhodes D, Nolte KD, Hicks WA, Leustek T, Cooper AJL, Hanson AD (1997) A new route for synthesis of dimethylsulphoniopropionate in marine algae. Nature **387:** 891-894

Galili G, Höfgen R (2002) Metabolic engineering of amino acids and storage proteins in plants. Metab Eng **4:** 3-11

Gibson JAE, Swadling KM, Burton HR (1996) Acrylate and dimethylsulfoniopropionate (DMSP) concentrations during an antarctic phytoplankton bloom.: new sources of reduced sulfur compounds. *In* RP Kiene, PT Visscher, MD Keller, GO Kirst, eds, Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds. Plenum Press, New York, NY, pp 213-222

Gil V, MacLeod AJ (1980) Studies of glucosinolate degradation in *Lepidium sativum* seed extracts. Phytochemistry **19:** 1369-1374

Giovanelli J, Mudd SH, Datko AH (1980) Sulfur amino acids in plants. *In* BJ Miflin, ed, The Biochemistry of Plants, Vol 5: Amino Acids and Derivatives. Academic Press, New York, NY, pp 453-505

Giovanelli J, Mudd SH, Datko AH (1985) Quantitative analysis of pathways of methionine metabolism and their regulation in *Lemna*. Plant Physiol **78:** 555-560

Giovanelli J, Mudd SH, Datko AH (1989) Regulatory structure of the biosynthetic pathway for the aspartate family of amino acids in *Lemna paucicostata* Hegelm. 6746, with special reference to the role of aspartokinase. Plant Physiol **90:** 1584-1599

Gómez-Gómez L, Carrasco P (1998) Differential expression of the *S*-adenosyl-L -methionine synthase genes during pea development. Plant Physiol **117:** 397-405

Graser G, Schneider B, Oldham NJ, Gershenzon J (2000) The methionine chain elongation pathway in the biosynthesis of glucosinolates in *Eruca sativa* (Brassicaceae). Arch Biochem Biophys **378:** 411-419

Gröne T, Kirst GO (1991) Aspects of dimethylsulfoniopropionate effects on enzymes isolated from the marine phytoplankter *Tetraselmis subcordiformis* (Stein). J Plant Physiol **138:** 85-91

Grossfeld RM, Yancey SW, Baxter CF (1984) Inhibitors of crayfish glutamic acid decarboxylase. Neurochem Res **9:** 947-963

Guthrie HA, Picciano MF (1995) Human Nutrition. Mosby, St. Louis, MO

Halsted CH, Villanueva JA, Devlin AM, Niemela O, Parkkila S, Garrow TA, Wallock LM, Shigenaga MK, Melnyk S, James SJ (2002) Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. Proc Natl Acad Sci USA **99:** 10072-10027

Hanson AD, Gage DA (1996) 3-Dimethylsulfoniopropionate biosynthesis and use by flowering plants. *In* RP Kiene, PT Visscher, MD Keller, GO Kirst, eds, Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds. Plenum Press, New York, NY, pp 75-86

Hanson AD, Rathinasabapathi B, Rivoal J, Burnet M, Dillon MO, Gage DA (1994a) Osmoprotective compounds in the Plumbaginaceae: a natural experiment in metabolic engineering of stress tolerance. Proc Natl Acad Sci USA **91:** 306-310

Hanson AD, Rivoal J, Paquet L, Gage, DA (1994b) Biosynthesis of 3-dimethylsulfoniopropionate in *Wollastonia biflora* (L.) DC.: evidence that *S*-methylmethionine is an intermediate. Plant Physiol **105:** 103-110

Hanson AD, Trossat C, Nolte KD, Gage DA (1997) 3-Dimethylsulphoniopropionate biosynthesis in higher plants. *In* WJ Cram, LJ De Kok, I Stulen, C Brunold, H Rennenberg, eds, Sulphur Metabolism in Higher Plants. Bauckhuys Publishers, Leiden, Netherlands, pp 147-154

Hatton AD, Malin G, Turner SM, Liss PS (1996) DMSO: A significant compound in the biogeochemicl cycle of DMS. *In* RP Kiene, PT Visscher, MD Keller, GO Kirst, eds, Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds. Plenum Press, New York, NY, pp 405-412

Haughn GW, Davin L, Giblin M, Underhill EW (1991) Biochemical genetics of plant secondary metabolites in *Arabidopsis thaliana*: the glucosinolates. Plant Physiol **97:** 217-226

Hell R (1997) Molecular physiology of plant sulfur metabolism. Planta **202:** 138-148

Holmberg N, Bülow L (1998) Improving stress tolerance in plants by gene transfer. Trends Plant Sci **3:** 61-66

Hysmith RM, Boor PJ (1988) Purification of benzylamine oxidase from cultured porcine aortic smooth muscle cells. Biochem Cell Biol **66:** 821-829

Inaba K, Fujiwara T, Hayashi H, Chino M, Komeda Y, Naito S (1994) Isolation of an *Arabidopsis thaliana* mutant, *mto1*, that overaccumulates soluble methionine: Temporal and spatial patterns of soluble methionine accumulation. Plant Physiol **104:** 881-887

James F, Nolte KD, Hanson AD (1995a) Purification and properties of *S*-adenosyl-L-methionine:L-methionine *S*-methyltransferase from *Wollastonia biflora* leaves. J Biol Chem **270:** 22344-22350

James F, Paquet L, Sparace SA, Gage DA, Hanson AD (1995b) Evidence implicating dimethylsulfoniopropionaldehyde as an intermediate in dimethylsulfoniopropionate biosynthesis. Plant Physiol **108:** 1439-1448

Janes SM, Palcic MM, Scaman CH, Smith AJ, Brown DE, Dooley DM, Mure M, Klinman JP (1992) Identification of topaquinone and its consensus sequence in copper amine oxidases. Biochemistry **31:** 12147-12154

John RA (1995) Pyridoxal phosphate-dependent enzymes. Biochim Biophys Acta **1248:** 81-96

Karsten U, Kück K, Vogt C, Kirst GO (1996) Dimethylsulfoniopropionate production in phototrophic organisms and its physiological function as a cryoprotectant. *In* RP Kiene, PT Visscher, MD Keller, GO Kirst, eds, Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds. Plenum Press, New York, NY, pp 143 -153

Katz ML, Gerhardt KO (1990) Storage protein in hereditary ceroid-lipofuscinosis contains *S*-methylated methionine. Mech Ageing Dev **53:** 277-290

Keller MD, Bellows WK, Guillard RRL (1989) Dimethyl sulfide production in marine phytoplankton. *In* ES Saltzman, WJ Cooper, eds, Biogenic Sulfur in the Environment. American Chemical Society, Washington, DC, pp 167-182

Keller MD, Korjeff-Bellows W (1996) Physiological aspects of the production of dimethylsulfoniopropionate (DMSP) by marine phytoplankton. *In* RP Kiene, PT Visscher, MD Keller, GO Kirst, eds, Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds. Plenum Press, New York, NY, pp 131-142

Kiene RP, Linn LJ (2000) Distribution and turnover of dissolved DMSP and its relationship with bacterial production and dimethylsulfide in the Gulf of Mexico. Limnol Oceanogr **45:** 849-861

Kim J, Lee M, Chalam R, Martin MN, Leustek T, Boerjan W (2002) Constitutive overexpression of cystathionine γ-synthase in Arabidopsis leads to accumulation of soluble methionine and *S*-methylmethionine. Plant Physiol **128:** 95-107

Klinman JP (1996) New quinocofactors in eukaryotes. J Biol Chem **271:** 27189-27192

Kocsis MG, Hanson AD (2000) Biochemical evidence for two novel enzymes in the biosynthesis of 3-dimethylsulfoniopropionate in *Spartina alterniflora*. Plant Physiol **123:** 1153-1161

Kocsis MG, Nolte KD, Rhodes D, Shen TL, Gage DA, Hanson AD (1998) Dimethylsulfoniopropionate biosynthesis in *Spartina alterniflora*: evidence that *S*-methylmethionine and dimethylsulfoniopropylamine are intermediates. Plant Physiol **117:** 273-281

Kocsis, MG, Ranocha, P, Gage, DA, Simon, ES, Rhodes, D, Peel, GJ, Mellema, S, Saito, K, Awazuhara, M, Li, C, Meeley, RB, Tarczynski, MC, Wagner, C, Hanson, AD (2003) Insertional inactivation of the methionine *S*-methyltransferase gene eliminates the *S*-methylmethionine cycle and increases the methylation ratio. Plant Physiol **131:** 1808-1815

Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an insertional mutagen in Arabidopsis. Plant Cell **11:** 2283-2290

Kumar A, Altabella T, Taylor MA, Tiburcio AF (1997) Recent advances in polyamine research. Trends Plant Sci **2:** 124-130

Laber B, Maurer W, Hanke C, Grafe S, Ehlert S, Messerschmidt A, Clausen T (1999) Characterization of recombinant *Arabidopsis thaliana* threonine synthase. Eur J Biochem **263:** 212-221

Lavine TF, Floyd NF, Cammaroti MS (1954) The formation of sulfonium salts from alcohols and methionine in sulfuric acid. J Biol Chem **207:** 107-117

Lee J-H, Chae HS, Lee J-H, Hwang B, Hahn KW, Kang BG, Kim WT (1997) Structure and expression of two cDNAs encoding *S*-adenosyl-L-methionine synthetase of rice (*Oryza sativa* L.). Biochim Biophys Acta **1354:** 13-18

Leustek T, Saito K (1999) Sulfate transport and assimilation in plants. Plant Physiol **120:** 637-643

Low PS (1985) Molecular basis of the biological compatibility of nature's osmolytes. *In* R Gilles, M Gilles-Baillien, eds, Transport Processes, Iono- and Osmoregulation. Springer-Verlag, Berlin, Germany, pp 469-477

Lugli J, Gaziola SA, Azevedo RA (2000) Effects of calcium, *S*-adenosylmethionine, *S*-(2-aminoethyl)-L-cysteine, methionine, valine and salt concentration on rice aspartate kinase isoenzymes. Plant Sci **150:** 51-58

Macnicol PK (1986) Analysis of *S*-methylmethionine and *S*-adenosylmethionine in plant tissue by a dansylation, dual-isotope method. Anal Biochem **158:** 93-97

Malin G (1996) The role of DMSP and DMS in the global sulfur cycle and climate regulation. *In* RP Kiene, PT Visscher, MD Keller, GO Kirst, eds, Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds. Plenum Press, New York, NY, pp 177-189

Malin G, Kirst GO (1997) Algal production of dimethylsulfide and its atmospheric role. J Phycol **33:** 889-896

Maloy OC (1993) Plant Disease Control: Principles and Practice. John Wiley and Sons, Inc., New York, NY

Mason TG, Blunden G (1989) Quaternary ammonium and tertiary sulfonium compounds of algal origin as alleviators of osmotic stress. Bot Mar **32:** 313-316

Mathews CK, van Holde KE (1990) Biochemistry. Benjamin/Cummings Publishing Company, Inc., Redwood City, CA

Matthews RG, Sheppard C, Goulding C (1998) Methylenetetrahydrofolate reductase and methionine synthase: biochemistry and molecular biology. Eur J Pediatr **157:** S54 -S59

Mayer RR, Cherry JH, Rhodes D (1990) Effects of heat shock on amino acid metabolism of cowpea cells. Plant Physiol **94:** 796-810

McCue KF, Hanson AD (1992) Salt-inducible betaine aldehyde dehydrogenase from sugar beet: cDNA cloning and expression. Plant Mol Biol **18:** 1-11

McNeil SD, Nuccio ML, Hanson AD (1999) Betaines and related osmoprotectants: Targets for metabolic engineering of stress resistance. Plant Physiol **120:** 945-949

Meeley RB, Briggs SP (1995) Reverse genetics for maize. Maize Genet Coop Newslett **69:** 67-82

Moffatt BA, Stevens YY, Allen MS, Snider JD, Pereira LA, Todorova MI, Summers PS, Weretilnyk EA, Martin-McCaffrey L, Wagner C (2002) Adenosine kinase deficiency is associated with developmental abnormalities and reduced transmethylation. Plant Physiol **128:** 812-821

Monaco TJ, Weller SC, Ashton FM (2002) Weed Science: Principles and Practices, 4th edn. John Wiley and Sons, Inc., New York, NY

Morris JT, Haley C, Krest R (1996) Effects of sulfide on growth and dimethylsulfoniopropionate (DMSP) concentration in *Spartina alterniflora*. *In* RP Kiene, PT Visscher, MD Keller, GO Kirst, eds, Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds. Plenum Press, New York, NY, pp 87-95

Mudd SH, Datko AH (1986) Methionine methyl group metabolism in *Lemna*. Plant Physiol **81:** 103-114

Mudd SH, Datko AH (1990) The *S*-methylmethionine cycle in *Lemna paucicostata*. Plant Physiol **93:** 623-630

Nellis DW (1994) Seashore Plants of South Florida and the Caribbean: A Guide to Identification and Propagation of Xeriscape Plants. Pineapple Press, Sarasota, FL

Nishiguchi MK, Somero GN (1992) Temperature- and concentration-dependence of compatibility of the organic osmolyte β-dimethylsulfoniopropionate. Cryobiology **29:** 118-124

Noh EW, Minocha SC (1994) Expression of a human *S*-adenosylmethionine decarboxylase cDNA in transgenic tobacco and its effects on polyamine biosynthesis. Transgenic Research **3:** 26-35

Nuccio ML, Rhodes D, McNeil SD, Hanson AD (1999) Metabolic engineering of plants for osmotic stress resistance. Curr Opin Plant Biol **2:** 128-134

Otte ML, Morris JT (1994) Dimethylsulphoniopropionate (DMSP) in *Spartina alterniflora* Loisel. Aquat Bot **48:** 239-259

Padiglia A, Medda R, Lorrai A, Murgia B, Pedersen JZ, Finazzi Agro A, Floris G (1998) Characterization of *Euphorbia characias* latex amine oxidase. Plant Physiol **117:** 1363-1371

Paquet L, Lafontaine PJ, Saini HS, James F, Hanson AD (1995) Évidence en faveur de la présence du 3-diméthylsulfoniopropionate chez une large gamme d'Angio-spermes. Can J Bot **73:** 1889-1896

Paquet L, Rathinasabapathi B, Saini H, Zamir L, Gage DA, Huang Z-H, Hanson AD (1994) Accumulation of the compatible solute 3-dimethylsulfoniopropionate in sugarcane and its relatives, but not other gramineous crops. Aust J Plant Physiol **21:** 37 -48

Pascale R, Simile M, De Miglio M, Feo F (2002) Chemoprevention of hepatocarcinogenesis: *S*-adenosyl-L-methionine. Alcohol **27:** 193-198

Pimenta MJ, Kaneta T, Larondelle Y, Dohmae N, Kamiya Y (1998) *S*-Adenosyl-L -methionine:L-methionine *S*-methyltransferase from germinating barley. Plant Physiol **118:** 431-438

Pokorny M, Marčenko E, Keglevič D (1970) Comparative studies of L- and D-methionine metabolism in lower and higher plants. Phytochemistry **9:** 2175-2188

Raamsdonk LM, Teusink B, Broadhurst D, Zhang N, Hayes A, Walsh MC, Berden JA, Brindle KM, Kell DB, Rowland JJ, Westerhoff HV, van Dam K, Oliver SG (2001) A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. Nat Biotechnol **19:** 45-50

Ranocha P, Bourgis F, Ziemak MJ, Rhodes D, Gage DA, Hanson AD (2000) Characterization and functional expression of cDNAs encoding methionine-sensitive and -insensitive homocysteine *S*-methyltransferases from *Arabidopsis*. J Biol Chem **275:** 15962-15968

Ranocha P, McNeil SD, Ziemak MJ, Li C, Tarczynski MC, Hanson AD (2001) The *S*-methylmethionine cycle in angiosperms: ubiquity, antiquity and activity. Plant J **25:** 575-584

Rathinasabapathi B, McCue KF, Gage DA, Hanson AD (1994) Metabolic engineering of glycine betaine synthesis: plant betaine aldehyde dehydrogenases lacking typical transit peptides are targeted to tobacco chloroplasts where they confer betaine aldehyde resistance. Planta **193:** 155-162

Ravanel S (1997) Methionine biosynthesis in higher plants: biochemical and molecular characterization of the transsulfuration pathway enzymes. CR Acad Sci Paris **320:** 497 -504

Ravanel S, Gakière B, Job D, Douce R (1998) The specific features of methionine biosynthesis and metabolism in plants. Proc Natl Acad Sci USA **95:** 7805-7812

Raven JA (1987) The application of mass spectrometry to biochemical and physiological studies. *In* DD Davies, ed, The Biochemistry of Plants: A Comprehensive Treatise, Vol 13: Methodology. Academic Press, San Diego, CA, pp 127-180

Raven PH, Johnson GB (1986) Biology. Times Mirror/Mosby College Publishing, St. Louis, MO

Recsei PA, Snell EE (1984) Pyruvoyl enzymes. Annu Rev Biochem **53:** 357-387

Reed RH (1983) Measurement and osmotic significance of β-dimethylsulphoniopropionate in marine macroalgae. Mar Biol Lett **4:** 173-181

Rennenberg H (1991) The significance of higher plants in the emission of sulfur compounds from terrestrial ecosystems. *In* TD Sharkey, EA Holland, HA Mooney, eds, Trace Gas Emissions by Plants. Academic Press, San Diego, CA, pp 217-260

Rhodes D, Gage DA, Cooper AJL, Hanson AD (1997) *S*-Methylmethionine conversion to dimethylsulfoniopropionate: evidence for an unusual transamination reaction. Plant Physiol **115:** 1541-1548

Rhodes D, Handa S, Bressan RA (1986) Metabolic changes associated with adaptation of plant cells to water stress. Plant Physiol **82:** 890-903

Rhodes D, Hanson AD (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu Rev Plant Physiol Plant Mol Biol **44:** 357-384

Roje S, Chan SY, Kaplan F, Raymond RK, Horne DW, Appling DR, Hanson AD (2002) Metabolic engineering in yeast demonstrates that *S*-adenosylmethionine controls flux through the methylenetetrahydrofolate reductase reaction *in vivo*. J Biol Chem **277:** 4056-4061

Hanson AD, Roje S (2001) One-carbon metabolism in higher plants. Annu Rev Plant Physiol Plant Mol Biol **52:** 119-137

Rontein D, Basset G, Hanson AD (2002) Metabolic engineering of osmoprotectant accumulation in plants. Metab Eng **4:** 49-56

Ross MA, Lembi CA (1999) Applied Weed Science, 2nd edn. Prentice Hall, Inc., Upper Saddle River, NJ

Russell DW, Howard AG (1996) The determination of DMSP in marine algae and salt marsh plants. *In* RP Kiene, PT Visscher, MD Keller, GO Kirst, eds, Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds. Plenum Press, New York, NY, pp 155-163

Salisbury FB, Ross CW (1985) Plant Physiology, 3rd edn. Wadsworth, Belmont, CA

Sandmeier E, Hale TI, Christen P (1994) Multiple evolutionary origin of pyridoxal-5' -phosphate-dependent amino acid decarboxylases. Eur J Biochem **221:** 997-1002

Schlenk F (1965) Biochemical and cytological studies with sulfonium compounds. *In* SK Shapiro, F Schlenk, eds, Transmethylation and Methionine Biosynthesis. University of Chicago Press, Chicago, IL, pp 48-65

Schmutz D, Brunold C (1984) Intercellular localization of assimilatory sulfate reduction in leaves of *Zea mays* and *Triticum aestivum*. Plant Physiol **74:** 866-870

Schröder P (1993) Plants as sources of atmospheric sulfur. *In* LJ De Kok, I Stulen, H Rennenberg, C Brunold, WE Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic Publishing, Hague, Netherlands, pp 253-270

Shen B, Li CJ, Tarczynski MC (2002) High free-methionine and decreased lignin content result from a mutation in the Arabidopsis *S*-adenosyl-L-methionine synthetase 3 gene. Plant J **29:** 371-380

Smith TA (1985) The di- and poly-amine oxidases of higher plants. Biochem Soc Trans **13:** 319-322

Steinke M, Daniel C, Kirst GO (1996) DMSP lyase in marine macro- and microalgae: intraspecific differences in cleavage activity. *In* RP Kiene, PT Visscher, MD Keller, GO Kirst, eds, Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds. Plenum Press, New York, NY, pp 317-324

Steudler PA, Peterson BJ (1984) Contribution of gaseous sulphur from salt marshes to the global sulphur cycle. Nature **311:** 455-457

Stevenson DE, Akhtar M, Gani D (1990) L-Methionine decarboxylase from *Dryopteris filix-mas*: purification, characterization, substrate specificity, abortive transamination of the coenzyme, and stereochemical courses of substrate decarboxylation and coenzyme transamination. Biochemistry **29:** 7631-7647

Storey R, Gorham J, Pitman MG, Hanson AD, Gage D (1993) Response of *Melanthera biflora* to salinity and water stress. J Exp Bot **44:** 1551-1560

Strange RN (1993) Plant Disease Control: Towards Environmentally Acceptable Methods. Chapman and Hall, London, UK

Stryer L (1988) Biochemistry, 3rd edn. W.H. Freeman and Company, New York, NY

Summers PS, Nolte KD, Cooper AJL, Borgeas H, Leustek T, Rhodes D, Hanson AD (1998) Identification and stereospecificity of the first three enzymes of 3-dimethylsulfoniopropionate biosynthesis in a chlorophyte alga. Plant Physiol **116:** 369-378

Sunarpi, Anderson JW (1997) Inhibition of sulphur redistribution into new leaves of vegetative soybean by excision of the maturing leaf. Physiol Plant **99:** 538-545

Sunda W, Kieber DJ, Kiene RP, Huntsman S (2002) An antioxidant function for DMSP and DMS in marine algae. Nature **418:** 317-320

Suzuki Y (1996) Purification and characterization of diamine oxidase from *Triticum aestivum* shoots. Phytochemistry **42:** 291-293

Suzuki Y, Hagiwara M (1993) Purification and characterization of diamine oxidase from *Zea mays* shoots. Phytochemistry **33:** 995-998

Tagmount A, Berken A, Terry N (2002) An essential role of *S*-adenosyl-L-methionine: L-methionine *S*-methyltransferase in selenium volatilization by plants: methylation of selenomethionine to *Se*-methyl-L-*Se*-methionine, the precursor of volatile selenium. Plant Physiol **130:** 847-856

Taylor BF, Kiene RP (1989) Microbial metabolism of dimethyl sulfide. *In* ES Saltzman, WJ Cooper, eds, Biogenic Sulfur in the Environment. American Chemical Society, Washington, DC, pp 202-221

Taylor BF, Visscher PT (1996) Metabolic pathways involved in DMSP degradation. *In* RP Kiene, PT Visscher, MD Keller, GO Kirst, eds, Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds. Plenum Press, New York, NY, pp 265-276

Thompson, JD (1991) The biology of an evasive plant: what makes *Spartina anglica* so successful? BioScience **41:** 393-401

Tolbert NE, Benker C, Beck E (1995) The oxygen and carbon dioxide compensation points of C_3 plants: possible role in regulating atmospheric oxygen. Proc Natl Acad Sci USA **92:** 11230-11233

Trethewey RN (2001) Gene discovery via metabolic profiling. Curr Opin Biotechnol **12:** 135-138

Trossat C, Nolte KD, Hanson AD (1996) Evidence that the pathway of dimethylsulfoniopropionate biosynthesis begins in the cytosol and ends in the chloroplast. Plant Physiol **111:** 965-973

Trossat C, Rathinasabapathi B, Hanson, AD (1997) Transgenically expressed betaine aldehyde dehydrogenase efficiently catalyzes oxidation of dimethylsulfoniopropionaldehyde and ω-aminoaldehydes. Plant Physiol **113:** 1457-1461

Trossat C, Rathinasabapathi B, Weretilnyk EA, Shen T-L, Huang Z-H, Gage DA, Hanson AD (1998) Salinity promotes accumulation of dimethylsulfoniopropionate and its precursor *S*-methylmethionine in chloroplasts. Plant Physiol **116:** 165-171

Turner SM, Malin G, Liss PS (1989) Dimethyl sulfide and (dimethylsulfonio)propionate in European coastal and shelf waters. *In* ES Saltzman, WJ Cooper, eds, Biogenic Sulfur in the Environment. American Chemical Society, Washington, DC, pp 183-200

Vianney P, Pocard J-A, Hamelin J, Blanco C, Bernard T (1998) Differential effects of dimethylsulfoniopropionate, dimethylsulfonioacetate, and other *S*-methylated compounds on the growth of *Sinorhizobium meliloti* at low and high osmolarities. Appl Environ Microbiol **64:** 1420-1429

Voet D, Voet JG (1995) Biochemistry, 2nd edn. John Wiley and Sons, Inc., New York, NY

Vojtěchová M, Hanson AD, Muños-Clares RA (1997) Betaine-aldehyde dehydrogenase from amaranth leaves efficiently catalyzes the NAD-dependent oxidation of dimethylsulfoniopropionaldehyde to dimethylsulfoniopropionate. Arch Biochem Biophys **337:** 81-88

Wakeham SG, Dacey WH (1989) Biogeochemical cycling of dimethyl sulfide in marine environments. *In* ES Saltzman, WJ Cooper, eds, Biogenic Sulfur in the Environment. American Chemical Society, Washington, DC, pp 152-166

Weigel P, Weretilnyk EA, Hanson AD (1986) Betaine aldehyde oxidation by spinach chloroplasts. Plant Physiol **82:** 753-759

Weretilnyk EA, Hanson AD (1989) Betaine aldehyde dehydrogenase from spinach leaves: purification, *in vitro* translation of mRNA, and regulation by salinity. Arch Biochem Biophys **271:** 56-63

Weretilnyk EA, Hanson AD (1990) Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. Proc Natl Acad Sci USA **87:** 2745-2749

White RH (1982) Analysis of dimethyl sulfonium compounds in marine algae. J Mar Res **40:** 529-536

Whitney EN, Hamilton EMN, Rolfes SR (1990) Understanding Nutrition, 5th edn. West Publishing Company, St. Paul, MN

Wink, M (1997) Special Nitrogen Metabolism. *In* PM Dey, JB Harborne, eds, Plant Biochemistry. Academic Press, San Diego, CA, pp 439-486

Wolfe GV, Steinke M, Kirst GO (1997) Grazing-activated chemical defense in a unicellular marine alga. Nature **387:** 894-897

Xiong H, Stanley BA, Tekwani BL, Pegg AE (1997) Processing of mammalian and plant *S*-adenosylmethionine decarboxylase proenzymes. J Biol Chem **272:** 28342-28348

Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. Ann Rev Plant Physiol **35:** 155-189

Zappia V, Zydek-Cwick CR, Schlenk F (1969) The specificity of *S*-adenosylmethionine derivatives in methyl transfer reactions. J Biol Chem **244:** 4499-4509

BIOGRAPHICAL SKETCH

Michael, the eldest of four children, was born in the southern Alberta, Canada, city of Lethbridge, into a family of irrigation farmers. Both Michael's parents were the children of farmers who had in the early part of the 20th century immigrated from Hungary. Due to the isolation that was often part of farming on the Canadian prairies, Michael's grandparents had to teach themselves how to speak and write English. Often, to facilitate communication, Hungarian was the language spoken at home. Michael's parents understood the value of a good English education, however, and always encouraged him, and his siblings, to study hard. After the death of Michael's biological father, his mother married a farmer who also had four children. Soon after the marriage, Michael's new and much larger family moved to the Peace River area of Alberta to farm.

From a very early age Michael had a great interest in agriculture. His interests went beyond the skills required to be a successful farmer, however. Michael was always interested in how plants were able to use nothing more than the sun's energy to convert nutrients found in the environment into the materials needed for growth and development. As a result of this curiosity, Michael started out on a life-long quest to determine how plants work, and how this knowledge could be exploited in a way that would improve farming and help feed the world's ever increasing human population.

Michael graduated high school from Spirit River Secondary School in Alberta, Canada, with a matriculation diploma. During high school and for a short time afterward Michael was involved in the business of agriculture, on the family farm and through

employment with a number of agricultural businesses. During his university studies Michael was employed by the Alberta Forest Service and an agri-chemical company, where he acquired invaluable practical experience in silviculture and agriculture. Later, Michael also worked for Agriculture and Agri-Food Canada and in university laboratories.

Michael's formal post-secondary education begins with a bachelor's degree in biological sciences from the University of Lethbridge, Alberta, Canada. Later he went on to receive a master's degree in biochemistry (Department of Chemistry) from the same institution. His studies continued to focus more specifically on plant metabolism and molecular physiology. Michael's formal education culminates with a doctoral (Ph.D) degree in plant molecular and cellular biology from the University of Florida, Gainesville, U.S.A.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Eminent Scholar of Plant Molecular and Cellular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kenneth Cline
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